Stereo Investigator®

USERS GUIDE

VERSION 10

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Table of Contents

Introducing Stereo Investigator 10 1
- What Is Stereo Investigator? 1
- What's New In Version 10? 1
- Getting Help 3
- Get Help from MBF Bioscience Support 4
- Training And Other Resources 5

Activating Your Software 7
- User Profiles and multiple users 8

Setting up the Workspace 11
- The Stereo Investigator Window, Toolbars, And Interface 11
- Informational Windows And Docking Markers 12
- Setting Up My Workspace 14
- Hardware Considerations 14

Working with Lenses 17
- Lenses: Installing And Calibrating 17
- Defining And Calibrating A New Lens 18
- Types Of Calibration 22
- Lucivid And Video Monitor Issues 23
- Parcentric And Parfocal Calibration 25
- Focus (Z-Step) Calibration 26
- Grid Tune Current Lens 28

Moving Around in Stereo Investigator 31
- Using The Joystick 31
- Aligning The Tracing And Specimen 32
- Moving Imported Images 33
- Working With AutoMove 34
- Working With Meander Scan 34

Contours and Tracing 37
- Tracing Contours 37
- Automatic Contouring 38
- Contour Measurements 41
- Editing Mode 45
- Selecting And Acting On Objects 46
- Hidden Objects 47
- Editing Contours And Points 47
- Editing Markers 51

Markers 55
- Marker Properties And Combination Markers 55

Automating Your Acquires 59
- Automating Your Work 59

The Image Stack Module 65
- Image stacks and lenses 68
- Image order and nomenclature 68
- Viewing image stacks 68

Multiple adjacent image stacks 68
Saving image stacks..... 69
Tracing from image stacks 69
The Serial Section Manager..... 71
The Coordinate System 71
Preparing Tissues for Serial Section Reconstruction 72
The Serial Section Manager Dialog Box..... 73
Setting Up The Serial Section Manager And Tracing..... 74
Tracing Serial Sections..... 75
Aligning Serial Sections 76
Serial Sections And Imported Images..... 77
Using A Data Tablet With Serial Sections 78
Working With Serial Sections—Upside Down Mountings 78
The MRI Module 81
How do I load and view MRI images? 81
The Deconvolution Module..... 83
What is Deconvolution 83
What do I need to perform Deconvolution? 83
Deconvolving an Image 84
Working with Deconvolution settings 85
3D Visualization 87
The 3D Visualization Interface..... 87
Working with 3D Objects and Attributes..... 87
Work with tracing settings..... 89
Work with view settings 90
Work with rotation settings 90
The Image Montage Module..... 95
Image montaging and how it works 95
Loading images and image stacks 98
Creating image montages..... 100
Saving image montages..... 102
The Virtual Tissue Module 103
Uses for Virtual Tissue 103
Setting Up for a Virtual Tissue Acquire 103
First Steps to Acquire..... 105
The Virtual Tissue Compiler 113
Displaying and Saving Virtual Tissues 113
Tracing from Virtual Tissue 114
Zooming in and out of Virtual Tissue 114
The ApoTome Module 115
Setting up the ApoTome 115
Stereological Probes: Foundations 119
Estimating And Measuring With Stereo Investigator 119
What Kids Of Object Properties Can Stereo Investigator Estimate?..... 121
Background Concepts For Using Stereological Probes 121
Tissue Preparation For Stereological Probes..... 126
The Disector And Optical Disector 129
Designing An Optical Disector Probe..... 133

Stereological Probes 135
Menu Commands 219
File Menu 219
Edit Menu 231
Move Menu 235
Tools Menu 241
Acquisition Menu 252
Image Menu 265
Options Menu 284
Help Menu 305
Keyboard Shortcuts 308
Toolbars 310
Index 321

Chapter 1

Introducing Stereo Investigator 10

What Is Stereo Investigator?

Stereo Investigator provides all the tools necessary for advanced microscope control and image acquisition in brightfield, fluorescent, and confocal systems.

You can acquire images from multiple fields of view and create seamless image montages, known as virtual slides. Stereo Investigator also enables you to use a single high-quality image acquisition application across all of your microscopes, a feature particularly beneficial for core facilities.

Stereo Investigator can save large amounts of disk space with its high-quality JPEG 2000 compression of images and stacks. Stereo Investigator also enables time-lapse image acquisition over multiple channels.

With confocal microscopes from Zeiss, Olympus, Nikon, and Leica, Stereo Investigator is also custom-designed for seamless integration with the world's leading motorized stages and cameras. Stereo Investigator is the ideal application for research scientists who need to capture images in 2D, 3D, and 4D.

What's New In Version 10?

- Create 3D mosaics from image stacks from confocal, brightfield, and fluorescent microscopes. Automatically or manually combine multiple image stacks to create a single composite image for analysis.
- New support for confocal microscopes.
    - Our new Open Confocal technology integrates with most major confocal microscopes to perform unbiased stereology. Acquire and analyze 3D images with Zeiss, Olympus, Nikon, and Leica laser scanning confocal microscopes.
    - Our new High Level Confocal technology fully integrates with Zeiss LSM confocal laser scanning microscopes running Zen software, so you can have the most advanced and streamlined confocal stereology solution available.
    - Allows easy creation of 3D montages of images and image stacks from virtually any confocal microscope.
- Full integration with the Zeiss ApoTome for confocal structured illumination. The leading cost-effective system for performing multichannel stereology.
- Stereo Investigator can now create focusable 3D virtual slides.

- Stereo Investigator now includes 3D visualization/solid modeling. Previously offered as an extension module.
- Fully integrated image deconvolution using the renowned Huygens technology increases image clarity for more effective analysis.
- Full 64-bit support for Zeiss Axiolmager, AxioCam and ApoTome; Olympus BX52 & BX-61 controllers (IX2/BX2 UCB & DSU); and Nikon 90i microscopes. Allows you to use the most sophisticated microscope automation for acquiring the largest images.
- Improved analysis (including Physical Fractionator, Cavalieri, etc.) of virtual slides from all major virtual slide scanner vendors.
- Improved Physical Fractionator with EM images.
- Automatic object detection on full virtual slides now applies to regions, cells, and markers.

New Hardware and File Support

- Full support for dual camera systems. Consistent image orientation for easier use of multi-camera systems, including QImaging, Hamamatsu, and Zeiss AxioCam cameras.
- Support for cameras using the DCAM interface protocol.
- Support for multimodal image acquisition. Allows you to create images, image stacks and virtual slides using a combination of fluorescent, confocal, darkfield and brightfield channels.
- Support for the Sutter Lambda, EXFO X-Cite 120PC, and X-Cite exacte Fluorescence Illumination Systems.
- Microlucida now reads Olympus OIB/OIF files.
- Enhanced support for the Nikon IDS/ICS format.
- Improved support for reading and writing XML files

Better productivity and Ease of Use

- Easily crop image stacks to reduce file size.
- Improved image, including selection and adjustment of multiple images. Easier and quicker operation, processing hundreds of images simultaneously.
- Select and adjust multiple objects. Selectively brighten images in a particular area or optimize images from a previously dark image acquire.
- Individually optimize the white and black point of each image in a stack to compensate for photobleaching and light attenuation.
- New tools for managing differential brightness across sections when acquiring images and image stacks.
- Use the Macro View to select your image in the Image Organizer.
- Improved 3D visualization.

- Use six times fewer mouse clicks while painting in Cavalieri mode or using Nucleator.
- Dynamic visualization of SRS image stacks in 3D.
- Improved user profile capabilities to allow full customization for multiple users of the to capture detailed images. Easier to obtain images for publication.

Getting Help

How do I get Help?

We designed Stereo Investigator and the Help to be easy to use and access. You can get help in the following ways:

- Press F1 to display the Help window. From there, you can use the Table of Contents pane, or the Index or Search tabs to find information.
- Click a Help button or icon. Some dialogs and windows have a Help button or blue Help icon.
- Clicking on one of these displays the Help topics associated with that dialog or window.
- Click an item in the Help menu.

Where Can I Find The README File?

Each time we release an updated version of the software, we include an updated README file. The README file contains late-breaking changes or information that could not be included into the Help file, as well as other information about this release.

Tell me about the Help window

The first time you use Help, the online Help window appears in a default location and size on your screen. You can change the way the Help window is displayed. After that, the Help window "remembers" its size and position.

Change the Size Or Position of the Help Window

- In the main window of Stereo Investigator, press F1 to open Help.
- To resize the Help window, move the pointer over a corner of the Help window until you see the double-headed arrow, and then drag the corner until the window is the size that you want it to be.
- To move the Help window, move the pointer to the title bar, and drag the window where you want it.

Mark the topic so I can come back to it later

If you need to refer to a topic often, you can add it to a list of your Favorites. This list is always available in the Tabs area in the left side of the Help window.

- While the topic is open, click the Favorites tab.
- Click the Add button.
  The Help system adds the topic to your favorites list.

Copy or print the contents of the Help window

You can copy the contents of the Help window and include them in another document, email message, or any other text application.

- Highlight the desired text in the Help window.
- Right-click and choose Copy.

You can also right-click in the Help window and choose Select All if you haven't selected any text.

How do I find the right content?

We've tried to make each topic as complete and informative as possible. We've included some tools to help you quickly find the right information. These include:

- An index of each topic based on its keywords
- A search function that searches the full text of each topic
- Tables of Content that list the topics in an easy to understand order

In addition to these tools, we include Related Topics links at the end of many topics. These can point you to other topics that relate to the topic at hand. We've also included links to support and training resources.

If you are still having difficulty finding the information you need, please click the feedback link at the bottom of each topic and let us know how we can improve our documentation.

Print an online help topic

You can print Help topics to keep as a handy reference or to give to other users.

- Click the Printer icon at the top of the Help window.
- Choose the printer in the Print dialog box and then click Print.

Get Help from MBF Bioscience Support

We know how important it is to have everything working properly with minimal downtime. Time spent troubleshooting issues is time lost for research. Our support team includes staff neuroscientists as well as experts in microscopy, stereology, and image processing.

The MBF Support Center is for registered users. If you need help with registration, please call 1-802-288-9290 for assistance.

- Training—We provide regularly-scheduled courses for our software, and we can also provide training at your location. MBF is also proud to sponsor stereology courses and workshops that are

presented by the most respected academics, free of commercial affiliation. Click here for information on training classes.

- Personal phone and email support—We believe in providing personal assistance, and we give you the option of receiving support via email and/or telephone. When you call, you will speak to a person, not a machine, for help with all of our software and hardware products, and you can always get back in touch with the same person who answered your previous question.
- Live remote assistance—Using just your web browser, you can connect directly to an MBF support person who can show you on your own computer how to run the MBF software and can also diagnose problems. Click on the Live Support link within Stereo Investigator to connect to a live support professional.
- Tips and tutorials—Our web site contains tips and video tutorials by our scientists and developers, covering a wide array of subjects. We've also included some tutorials with the software. Click **Help>Tutorials** in Stereo Investigator.
- MBF KnowledgeBase—Our online support site provides instant, 24/7 detailed responses to common questions. Each answer in our Knowledge Base was supplied by our MBF experts in neuroscience, microscopy, and image analysis.

Training And Other Resources

Get Training From MBF Bioscience

MBF Bioscience offers personalized training from our expert staff at our Vermont offices. Tailored to your needs, we devote our resources to helping you understand how to use and to best leverage the power of our products applied to your research. Save time by learning hands-on the features of Stereo Investigator using your own tissue.

For more information, including course dates, contact MBF Bioscience or visit our training page.

Other resources

For a list of other resources that you might find useful, visit our Resources and Links page, which is frequently updated with new information.

More Online Resources

- MBF Bioscience has its own Facebook page.
- The MBF Bioscience Software Users Group on LinkedIn is another useful resource for students and professionals.
- We are on Twitter as MBFBioscience.
- We post instructional videos, news from trade shows, and other information on our Youtube channel.

Chapter 2

Activating Your Software

Authorizing My License

Authorization is the process that checks and verifies your license.

Your license is authorized by one of two methods:

- An Authorization key. You can obtain this key by contacting MBF Bioscience. This is the most common method.

  -or-

- A dongle, a small device that attaches to your computer's USB port. This method is used when you want to move your Stereo Investigator license among different computers.

Where Can I Find My License Information?

Stereo Investigator displays the license information in the **System Settings** dialog box.

Click **Help>System Settings** to view which modules you are licensed to use.

For the End User License Agreement (EULA), see the file MbfLicense.txt in your Stereo Investigator product directory.

Using Stereo Investigator With A Dongle

Your security dongle attaches to a USB port on your computer, and must be present for Stereo Investigator to operate. If you are using Stereo Investigator with a dongle, your software is already authorized for use, and you do not need to contact MBF Bioscience for authorization.

Your dongle is very important! If you lose your dongle, you must contact MBF Bioscience Product Support for a replacement.

Why Would I Use a Dongle?

If you want to use mobile licensing, you would use a dongle. That way, you can install Stereo Investigator on different computers and use the dongle to move the license from computer to computer.

What Are The Stereo Investigator Terms Of Use?

For the End User License Agreement (EULA), which constitutes the terms of use, see the file MbfLicense.txt in your Stereo Investigator product directory.

User Profiles and multiple users

The Stereo Investigator User Profiles command allows multiple users and groups to work with the software using their own unique settings and preferences. Don't confuse User Profiles with a login or validation feature—the Profile Manager makes it easier to copy and share profile settings.

Learn more about profiles

If you administer a lab with many users or if you share Stereo Investigator with someone else, you use the **User Profiles** to sign on to Stereo Investigator and to create, change, or remove user profiles. Each user profile is unique for each user, but profiles can have the same program settings. For example, administrators can pre-configure profiles with lenses, cameras, and other equipment used in their labs.

Profiles contain the following user information:

- Stereo Investigator. ini file—information Stereo Investigator needs to operate, including the settings and preferences you use
- Stereo Investigator. len—lens information and settings
- Stereo Investigator. UI—information on which toolbars you have on display and which windows, such as the Serial Section Manager, are open and where they are placed
- any configuration and data backup files

Profiles are a new feature with Stereo Investigator. If you are upgrading from an earlier release, you can use your old Stereo Investigator. ini and Stereo Investigator. len files. If any changes need to be made, Stereo Investigator makes the changes.

Create a new group

1. Click **Options>User** Profiles.
2. In the **Profile Manager** dialog box, click **New Profile.** Stereo Investigator displays the **Create New Profile** dialog box.
3. Click **New Group**.
4. Type a name for the group, such as *Neuroscience 232 Lab* or *Dr. Boswell's group.*
5. Click **OK.**

Create a new user

1. Click Options>User Profiles.
2. In the Create New Profile dialog box, click New Profile.
3. Type a name in the **Name** text box, and then click **OK.**

If you want to copy existing user settings, see **Import profile settings** on page 9.

Delete groups

If you delete a group, all that group's users are also deleted. Backup your group and user settings before deleting any groups or users.

1. Navigate to the Configuration folder. For example, *C:\Program Files\MBF Bioscience*\Stereo Investigator*Configuration*
2. Select the group you wish to delete and drag it to the Trash or right-click **Delete**.

When you empty the Trash, you are deleting all the settings for that group.

Delete users

1. Navigate to the Configuration folder. For example, *C:\Program Files\MBF Bioscience*\Stereo Investigator*Configuration*
2. Open the folder for the group that contains the user you want to delete.
3. Select the user you want to delete and drag it to the Trash or right-click **Delete**.

When you empty the Trash, you are deleting all the settings for that user.

Import profile settings

1. Click **Options>User Profiles.**
2. In the **Profile Manager** dialog box, click **New Profile**. Stereo Investigator displays the **Create New Profile** dialog box.
3. Click **Import**.
4. Select the product .ini file and click **Select**.
5. In the Create New Profile dialog box, type a name and click **OK**.
   Stereo Investigator creates the new profile with the imported settings.

Chapter

3

Setting up the Workspace

The Stereo Investigator Window, Toolbars, And Interface

Before plunging into using Stereo Investigator, take some time to explore the workspace and set it up the way you'd like. You can customize and configure the workspace to help make your work easier and more efficient.

The Tracing Window

Stereo Investigator takes full advantage of many of the advanced Windows interface features such as dockable toolbars and right mouse button menus. Take a moment to familiarize yourself with the basic features of the Stereo Investigator interface. The central window is referred to as the Tracing Window.

The Toolbars

The toolbars sit under the menus, but you can click on a toolbar and drag it anywhere on your display. Each of the toolbars can be turned off. If you hover the mouse pointer over a toolbar button, Stereo Investigator gives you a brief description of its function. To learn about each of the toolbars and buttons, see the topics under Toolbars and shortcuts in the Help.

The Markers Bar

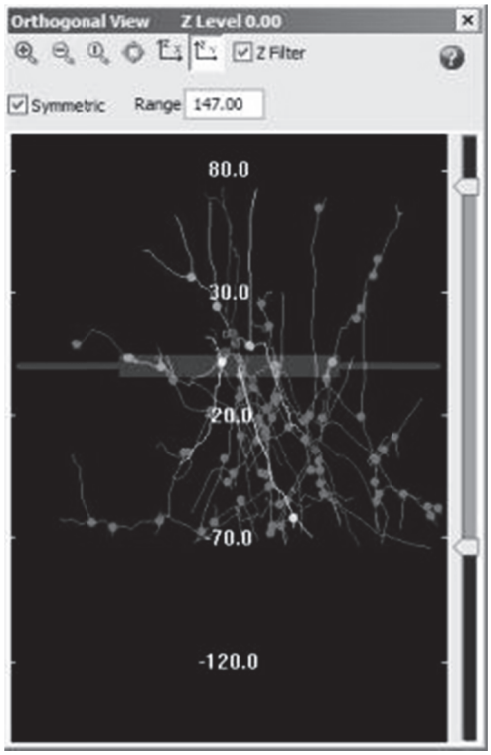
The Markers toolbar contains all the markers you can place in Stereo Investigator. Click a marker to select it, and then click on an item in the Tracing window to place the markers.

Informational Windows And Docking Markers

Stereo Investigator also includes informational windows such as **Orthogonal View, Macro View, Z Meter,** and the **Diagnostics** window to give you more information or an alternate view of your data. You can dock these windows by dragging them over the Docking Markers and releasing over the marker arrows. You can also move these windows anywhere on your monitor. The docking markers are visible when you drag an information/detail window.

Orthogonal View

The Orthogonal View eliminates the effect of distance from a viewpoint, and therefore provides a useful means of locating points and objects in 3-D space. Click in the image to learn about the **Orthogonal View** controls.



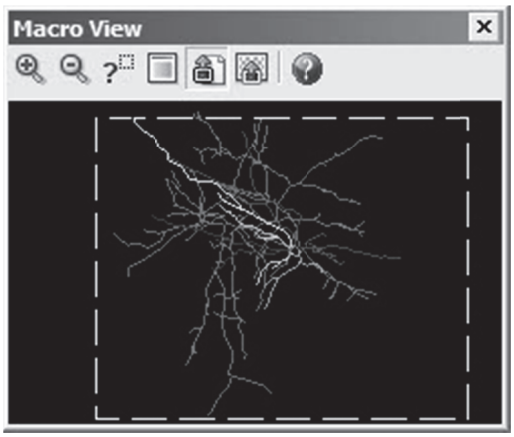
Contour Measurements

This windows shows you a list of contours and information about them. The window has the following buttons:

- **Equations**—Displays contour equation information.
- **Print**—Prints the contour measurements information.
- **Copy to Clipboard**—Copies the contour measurements information to the Windows Clipboard.
- **Close**—Closes the window.

Macro View

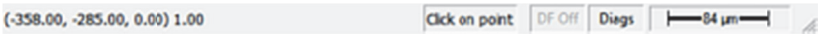
The **Macro View** shows you the entire work area, including the area outside the current view.



The Status Bar

The **Status Bar**, at the bottom of the screen, is divided into two sections:

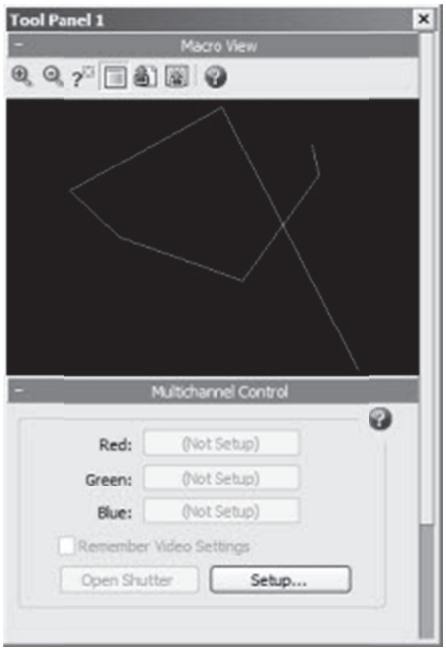
- The **Position Pane** (located in the left part of the status bar) contains the coordinates of the cursor in the format: (X,Y,Z) diameter (size). The X and Y values reflect the X and Y position of the cursor relative to the reference point. The Z value reflects the current focal depth within the current section. Diameter shows the size of the circular cursor, which controls the drawn thickness of contours and neuronal processes, and the diameter of drawn markers. Size shows the dimension of the crosshair cursor.



- The **Status Message Pane** displays important messages while you are working. These messages prompt you to perform actions as well as provide information about what the program is expecting you to do next.

Tool Panels

If there are controls, display windows, and other items that you often use and refer to, you can group them together in a tool panel. This tool panel groups these items; you can move or size the tool panel as a single entity. For instructions on working with tool panels, see **Options>Configure Tool Panels**.



Setting Up My Workspace

You can move many interface elements—toolbars, information and detail windows, the markers bar—anywhere inside the Stereo Investigator interface, or outside of it. If you are using two monitors, you can set up one as a tracing window, and keep your toolbars and other interface items on the secondary monitor.

Stereo Investigator remembers the position of these interface items when you exit the software, so you don't have to set it up each time.

Hardware Considerations

If you purchased a new system of Stereo Investigator and hardware from MBF Bioscience, your hardware has been properly installed, configured, and tested. If you are installing new hardware yourself, please read this section for some useful information.

WARNING! —In general, unless you are knowledgeable about these settings, it is a good idea to contact us before changing hardware configurations; this section is intended to let the you know how to access the configuration settings, and the pre-set configurations that are available.

Motorized Stages And Position Encoders

The default settings for many motorized stages and position encoders have been pre-programmed into the Stereo Investigator software. Click **Options>Stage Setup** and either the **Stage Type** or **XYZ-axis** tab on the dialog box and click **Use Defaults**.

- **Stage Type:** Supported motorized stage types, stage controllers, and encoders include those made by LUDL, Prior, Applied Scientific Instrumentation, Märzhauser, Boeckeler, and Zeiss. The list of supported stages is constantly upgraded to include the most commonly used motorized stages, so be sure that you have the most recent version of our software if your stage is not listed. If you have

a motorized stage or position encoder not included in the Stage Type list, please contact MBF Bioscience Product Support for assistance in configuring the system for your stage.

- **Z-axis:** Some motorized stage configurations incorporate integrated Z-axis (focus) position encoders; if this is the case, choose **No Separate Z-stage or external encoder** on the **Z-axis** tab. If you are using the internal focus motor of your Zeiss, Olympus, or Leica microscope, choose the appropriate microscope model from the list. We also support the external Heidenhain Z-ND 281 position readout with RS-232 interface.

If you are using Stereo Investigator *without* a microscope, with acquired images or virtual slides, choose **Manual Stage.** You may also set the Z-axis **to MBF Virtual Z Stage.**

Video Cards

You can use Stereo Investigator with several video capture (frame grabber) cards that display and acquire live or grabbed video images obtained from cameras. Click **Options>Video Setup** to see the list of supported cards and change settings. After choosing a video card, use the Settings tab to modify some of the operating parameters of the video card. These settings include the key color, X-Y position offsets, and hardware profile. Typically, you set these once. For more commonly accessed controls such as contrast, brightness, etc. click **Imaging>Adjust Camera Settings**.

Some generic video cards may also perform satisfactorily for working with live images, however, **Acquire Image** and other image processing commands do not operate properly. In order to work properly with Stereo Investigator software, a generic card must be able to maintain a live image when information is drawn over the video image without going into freeze frame mode.

Most frame grabbers need to have their manufacturer-supplied drivers installed and correctly configured before Stereo Investigator can use them correctly.

Video And Digital Cameras

Video cameras often include settings controlled by switches and external control boxes. Please consult the camera manufacturer's instructions before operating the video camera and its controller. If you are having trouble obtaining a live image in Stereo Investigator, the first step in troubleshooting is to ensure that the camera is turned on and set to its default configuration, and that the microscope is configured to send the light to the camera. Also check to be sure that you selected **Imaging>Live Image**. Turn on the color bars of the camera (if available) to check that the camera is able to send images to the computer. If color bars are visible but not a live image, this is usually because there is insufficient illumination of your tissue.

Unlike video cameras, digital cameras typically have no external controls to adjust; they are completely controlled by software. Click **Imaging>Adjust Camera Settings** to adjust the settings of digital cameras.

Cameras can be easily knocked out of alignment, so check alignment often. See Rotational alignment for details and instructions.

Lucivid

After turning on the Lucivid, and checking that light path settings are correct for viewing your specimen through the oculars, you may need to make further adjustments to the settings for an optimal viewing environment. Please see your Lucivid documentation for more information about the operation and adjustment of the Lucivid.

Chapter 4

Working with Lenses

Lenses: Installing And Calibrating

Proper calibration of all of your lenses is the only way your computer knows how many microns to assign to each pixel in the digital image on the screen. This allows for accuracy that you depend on in measurements, area and volume analysis, 3D reconstruction, and data analysis. Your physical lens and camera must be in correct physical alignment in order to maintain the positional correspondence between the tracing and the slide material.

Lens calibration is not difficult. Take the time to calibrate your lenses regularly. Proper lens calibration is the only way to assure that your measurements and data are accurate.

As a good rule of thumb, check and correct calibration whenever you start a new project.

Rotational Alignment

Correct physical alignment of the video camera where it attaches to the microscope is essential. **The rotational alignment of the system must be completed before calibrating the lenses.** This alignment is accurate until some component on the microscope is moved or changes. If a specimen and tracing are not properly aligned after an **AutoMove** or **Move**, check for errors in alignment or calibration.

To Check Rotational Alignment:

1. Place a slide with a distinct object on the microscope, then center and focus on the object. With the object in the tracing window, click anywhere in the tracing window to set a reference point.
2. Focus on the object.
3. Click **Options>Display Preferences>Grid** and check **Grid Enabled**. In the Grid Spacing box, select a grid size that gives a widely spaced grid with at least one horizontal line visible at all magnifications.
4. Click **Move>Joy Free** and use the joystick to align the object with one of the grid lines at either the far right or far left of the tracing window. Line up the top or bottom edge of the object with the grid line, rather than trying to center it.
5. Using the joystick, move the stage left to right along the X-axis. If the object visually drifts above or below the grid line you need to adjust the rotational alignment.

To Adjust Rotational Alignment

1. Loosen the setscrew that holds the video camera in place on the microscope so that the camera rotates in the holder as it is turned by hand, but not so loose that is spins freely.
2. Starting with the object at one end of the field of view, just touching one of the grid lines and move it all the way to the other side of the field of view.
3. While looking at the specimen on the monitor **gently** turn the camera so that you move the object about half way back to the grid line from its stopping position.
4. Move the stage in the Y-axis so the object is once again just touching one of the lines, and move back and forth in the X-axis to check alignment. Repeat this procedure until the object tracks perfectly along the horizontal grid line.
5. Tighten the setscrew and recheck the alignment. Often the act of tightening the screw alters the alignment slightly, so it may take a few tries to get perfect alignment. Try tightening the setscrew part way, making final adjustments, and then tightening the rest of the way.

To ensure best alignment, start with a high power lens, and then recheck with a low power lens. This checks the alignment over a greater path of X-axis movement.

Defining And Calibrating A New Lens

1. Start Stereo Investigator.
2. Choose the lowest power objective on the microscope turret.

   This procedure is for defining new lenses, so do not use the lenses listed in the Lens box.

3. Use the joystick to center the 250µm slide grid in the tracing window, and focus on the slide grid.
4. Click anywhere in the tracing window to place a reference point.

   If a reference point was already placed, click on the Joy Free button to enable joystick movement and center the grid. Exit Joy Free mode by clicking the button again.

5. Click **Tools>Define New Lens**. Stereo Investigator displays the **Define New Lens** dialog box.
6. Type a name for the current lens (10X, 25X, etc.)
   - **Lens Type:** Choose whether the lens is Optical,Video, or Tablet.
   - **Correction Factor:** Choose whether the lens is **Air, Oil, Water,** or **Other**. If it is **Other**, you need to enter a depth correction factor based on the refractive index to be applied to Z data

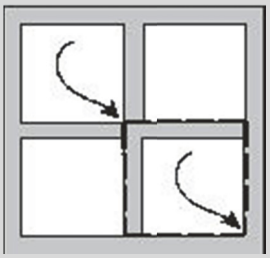
   Tell me about the correction factor: The reason for the correction factor is that Stereo Investigator has to calculate the location of the focal plane, as opposed to the position of the microscope. The location of the focal plane is dependent on the refractive index of the medium through which light is being transmitted, according to Snell's Law. A one micron change in the microscope position does not mean that the focal position changed one micron. There are also situations in which the default correction factors selected by using the air, oil, or water buttons are not the desired values. Although not common, it is important to be aware of this when calibrating a lens, and consider the possibility if your calibration seems off.

- **Calibration Box Setup:** Enter the calibration box size (250µm or 25µm if using the MicroBrightField graticule slide).

Force Square is only used with tablet lenses or images in which the scale bar provided allows for calibration in only one axis. Do not check this option when calibrating from a grid slide calibration squares.

7. Click **OK,** and follow the instructions in the status bar to draw a calibration box. The first point of the calibration box should be at the top left of a slide grid square. Click again in the lower right corner of the calibration box. Getting these initial clicks perfect is not essential, as fine tuning is the next step.

If the magnification is high enough that the grid slide lines are thick bars rather than lines, you will obtain the best results if you line up the cursor with one edge of the line, rather than trying to estimate where the precise middle of the line lies. Line up the calibration box with the upper edge of the horizontal lines and the left edge of the vertical lines. This puts the first point at the "Northwest corner" of the line intersection, as shown here.



Making the cursor larger helps to align it with the edges of the slide grid boxes. Use the up and down arrow keys to change the length of the lines of the cursor.

8. After outlining the calibration box, Stereo Investigator starts the **Grid Tune** operation. An anchor icon appears at the point of the top left corner of the calibration box, with a dashed line grid covering the rest of the window. This grid should roughly align with the grid on the calibration slide. If the anchor is not perfectly aligned with the vertex of the grid slide, drag it to the correct location (one of the intersection corners illustrated above).

9. Adjust the dashed line grid until it matches the slide grid perfectly. Use the cursor to move any of the dashed grid lines to tune the calibration. The best calibration is obtained when the dashed line furthest from the anchor point is moved to perfectly align with the grid squares at this location. Align the dashed line grid vertically and horizontally, getting the best possible correlation with the grid squares on your calibration slide.

To adjust the grid line spacing move the cursor over a line. The cursor changes to a sizing arrow that you click and drag to move the line. If the cursor is moved over an intersection of the dashed grid the cursor changes to a 4-way arrow indicating that the vertical and horizontal dimensions can be changed simultaneously. This works well for coarse adjustments. We recommend that vertical and horizontal adjustments be performed separately for best results.

10. When you have finished aligning the grid, right-click and select **Finish Calibrating Current Lens.**

Calibrating An Existing Lens

Lenses sometimes go out of calibration due to handling a lens in its turret, bumping or jarring your microscope, or for other reasons.

To Calibrate an Existing Lens

1. Click **Tools>Grid Tune Current Lens.**
2. In the **Grid Tune Current Lens dialog** box, make any changes to the **Box Size** and **Force Square** items, and then click **OK.** Stereo Investigator displays an anchor icon at the point of the top left corner of the calibration box, with a dashed line grid covering the rest of the window. This grid should roughly align with the grid on the calibration slide. If the anchor is not perfectly aligned with the vertex of the grid slice, click and drag it to one of the grid intersection corners.
3. Adjust the dashed line grid until it matches the slide grid perfectly. Use the cursor to move any of the dashed grid lines to tune the calibration. The best calibration is obtained when the dashed line furthest from the anchor point is moved to perfectly align with the grid squares at this location. Align the dashed line grid vertically and horizontally, getting the best possible correlation with the grid squares on your calibration slide.

   To adjust the grid line spacing move the cursor over a line. The cursor changes to a sizing arrow that you click and drag to move the line. If the cursor is moved over an intersection of the dashed grid the cursor changes to a 4-way arrow indicating that the vertical and horizontal dimensions can be changed simultaneously-- this works well for coarse adjustments. We recommended that vertical and horizontal adjustments be performed separately for best results.

4. When you have finished aligning the grid, right click and select **Finish Calibrating Current Lens.**

Checking Calibration

This method describes how to check calibration using the displayed grid and the calibration slide. To check calibration and make corrections at the same time, use the Grid Tune method described in To fine tune calibration.

The best way to check the calibration of your lenses is with the MicroBrightField calibration slide that is included with the MicroBrightField system. This slide has two grids of grids of 250µm and 25µm squares within a central area of the slide. The larger grid consists of a 16X16 grid of 250µm squares. Move left from the center of the larger grid to find the smaller grid, consisting of a 20X20 grid of 25µm squares.

Center the slide on the microscope, focus on one of the grids and use the following procedure to check your calibration. Make sure your camera is in rotational alignment before beginning.

In this section, reference is made to the slide grid (the grid on the MicroBrightField calibration slide or other calibration slide that is used) and to the dashed line grid (the grid generated by Stereo Investigator and displayed in the tracing window). The essence of calibration is to align these two grids.

1. Open Stereo Investigator.
2. Check that the lens in the turret matches the lens listed in the **Lens Selection** list box.
3. Using the joystick, center one of the slide grids. Use the 250µm or 25µm grid depending on the magnification of the objective you are using.
4. Click anywhere in the tracing window to set a reference point.

5. Click **Options>Display Preferences>Grid** and select **Enable Grid**. Choose a grid size (either 25µm or 250µm) that matches the size slide grid you are displaying in the tracing window.
6. Click **Joy Free** and use the joystick to line up the dashed line grid with the slide grid on the calibration slide. Line up a grid intersection near the top left corner of the tracing window. Note that in aligning the dashed line grid with the grid on the slide, the dashed line grid should line up with the "northwest" corner of the grid intersections on the glass slide.
7. Align the dashed line grid with the slide grid.
8. Check the grid lines in the bottom right of the tracing window to see if they are also lined up. If they line up perfectly, then your calibration is good for that lens. Repeat the above procedure for all lenses to be used. If the grids do not line up well, follow the instructions in To fine tune calibration.

To fine tune calibration

Use these instructions to make minor corrections to lenses previously defined that have errors in calibration. If the calibration of a lens is off by a great deal, delete the lens and redefine it as a new lens

1. Click **Tools>Grid Tune Current Lens** and enter the grid size you are using. A white dashed line grid appears with an anchor icon at one of the intersections. Ideally the size of the dashed line squares is roughly the same as the squares of the calibration slide.
2. Click and drag the anchor and align it over one of the vertices of the calibration slide grid.
3. Line up the grid with the edges of the slide grid at high magnification.
4. When the mouse is moved over any dashed line, it turns into a double-headed arrow, which enables moving that line. Use this arrow to move the dashed lines furthest from the anchor until they line up with the slide grid lines.
   Align both horizontal and vertical lines in this step. This should bring all lines of the grid into alignment with lines on the calibration slide. Once this alignment has been correctly adjusted, right click and choose **Finish Calibrating Current Lens**. Repeat with all lenses that are not properly calibrated, being careful that the lens in the **Lens Selection** list box matches the lens in the turret.

   It is possible that only the dashed lines in approximately the middle third of the screen can be lined up exactly with the lines on the calibration slide. This may be due to optical aberration in the objective lens (in which case the black lines may appear slightly curved), other optics in the microscope (such as a beam splitting prism) if you are viewing through the eyepieces. If this happens, just align the middle third of the grids, and don't worry about aligning the outer portion of the field. If you know you have this kind of optical aberration, and are doing work that requires precise measurements, you may want to set your **AutoMove** area to be the size of the area accurately calibrated, and only work within that area.

5. Place a marker very precisely on an object on the slide.
6. Move the object to another region of the tracing window with **Move To** or **Joy Track**. Following the move, the marker and specimen should still be in perfect registration.
7. Repeat these steps for each lens to be used, performing separate alignments for video and optical lenses.

Types Of Calibration

Calibration For Imported Images

When tracing from imported images, it is important to calibrate a lens to use with these images so that Stereo Investigator can provide accurate data about the tracings made from these images. There are a few ways to do this:

- If you acquired the image on your current system, simply choose the same lens that was used to acquire the image.
- If the image contains a scale bar, click **Tools>Define New Lens** to define a lens for these images. See Lenses: Installing and Calibrating for more information and instructions.
- To calibrate a lens for an imported image, the image must be at its 1:1 (100% zoom) resolution. If you need to zoom out to find the scale bar on the image, the image must be returned to 100% zoom before calibration is started. Use the Move Arrows or the **Macro View** window's **Go To** function to move to the region of the scale bar before calibrating the new lens.
- If you know the number of microns per pixel for the acquired image, click **Tools>Edit Lens**, and select a lens. In **the Edit Lens Parameters** dialog box, type the number of microns per pixel in the Scale Factor box.

Be sure you have calibrated a lens appropriately for each imported or acquired image, and be certain to have the right lens selected when tracing from any imported or acquired images!

Calibration For Macro Lenses

You may want to use a video camera mounted on a photo stand to trace from a photographic negative placed on a light box. Follow the procedure outlined below to complete the calibration.

1. Prepare a figure containing a calibrating square whose height and width corresponds to the scale bar for the photomicrograph to be traced.
2. Click **Imaging>Live Image**.
3. Place the square calibrating figure on the table so that the figure's sides are aligned with the X- and Y-axes of the Stereo Investigator graphics overlay.
4. Click **Tools>Define New Lens** and specify the appropriate information for this lens and the size of the calibration square. This lens should be specified as a Video lens, and its name should reflect as much scaling information as possible (camera lens, etc.).
5. Click **OK**.
6. Use the left mouse button to click once at the upper left-hand corner of the calibration box and then again at the lower right-hand corner. This completes the calibration.

Each time you change the magnification of the macro lens or adjust the height of the camera from the tablet, you need to recalibrate the lens.

Calibration For Data Tablets

If you are using a data tablet, you use **Tools>Define New Lens** command to calibrate the tablet. There are slight differences, however. If you are calibrating to trace an object placed on a data tablet, it is necessary to have a 2D scale that matches the object. This can be an "L" or a square.

1. Prepare a figure containing a calibrating square whose height and width corresponds to the scale bar for the photomicrograph you plan to trace.
2. Place this figure on the tablet so that its edges are aligned with the tablet's X- and Y-axes.
3. Set the data tablet to **Absolute** mode. Refer to the instructions for the data tablet in the manufacturer's manual for detail on how to do this.
4. Click **Tools>Define New Lens**.
5. Choose a lens name that reflects the scaling of the calibration square.
6. Enter the length of one side of the calibration square as the box size.
7. For the data acquisition method, click on the **Tablet** button
8. Align the crosshair of the data tablet mouse to the upper left-hand corner of the calibration box and click on the left mouse button (this should be the yellow button on the data tablet mouse).
9. Move the crosshair of the data tablet mouse to the lower right-hand corner of the calibrating box and click the left mouse button again. This completes the calibration.

When you are done tracing from the data tablet, remember to reset the data tablet to **Relative** mode. Refer to the instructions for the data tablet in the manufacturer's manual for detail on how to do this.

Lucivid And Video Monitor Issues

If both a Lucivid and a computer monitor with a video camera are being used to view the specimens and make tracings, it is very important to calibrate separate video and optical lenses. The scaling of the image through the oculars and through the video camera are almost sure to be different, and the X and Y step sizes that control stage movement are also different. If the same lens settings are used for both monitor and Lucivid tracing, the tracing and image are not properly aligned following stage movements.

Monitor Settings

The lenses that appear in the **Lens** box are only those that have been calibrated for the currently selected display resolution (640x480, 800x600, etc.). If you are using a different display resolution during data acquisition the lenses must be recalibrated at each resolution, and lens names should be assigned that differentiate between resolutions.

To check the resolution of your monitor, right-click on the **Windows** desktop, click on **Properties**, and choose the **Settings** tab. The **Screen Area** or **Screen Resolution** field shows your current resolution.

Checking And Adjusting Alignment

Correct physical alignment of the video camera or Lucivid. where it attaches to the microscope is essential to maintain the positional correspondence between the tracing and the material on the slide. The rotational alignment of the system must be completed before calibrating the lenses. Once alignment has

been performed, it remains accurate until some component on the microscope has been moved. However, these components are often inadvertently bumped in the course of regular use, so it is important to periodically check and correct the rotational alignment of the system.

If the tracing and specimen do not properly re-align after an **AutoMove** or **Move** function, the most likely cause is an error in calibration and/or alignment.

Rotational alignment can be performed using any slide, as long as it has a distinctive punctate object somewhere on it. This object can be a speck of dust, a small cell, or a chip on the slide. Place the slide on the microscope, center and focus on this object, then follow the next set of instructions to check and adjust the rotational alignment.

1. Start Stereo Investigator.
2. Use the joystick to move the stage so that the selected object appears in the tracing window. Click anywhere in the tracing window to set a reference point. Focus on the object.
3. Display a grid by selecting **Options>Display Preferences>Grid** and checking **Grid Enabled**. In the Grid Spacing box, select a grid size that gives a widely spaced grid with at least one horizontal line visible at all magnifications. The grid is needed for a straight horizontal line, so an alternative is to use one of the horizontal lines of the **AutoMove** box.
4. Select **Joy Free** and use the joystick to align the object with one of the grid lines (or a horizontal edge of the **AutoMove** box) at either the far right or far left of the tracing window. It is best to line up the top or bottom edge of the object with the grid line, rather than trying to center it.
5. Leave **Joy Free** activated, and use the joystick to move the stage left to right along the X-axis only. If the camera or Lucivid. is properly aligned, the object tracks along the grid line. If the object visually drifts above or below the grid line, the rotational alignment should be adjusted by following the instructions beginning in step 6.
6. Loosen the setscrew that holds the video camera or Lucivid. in place on the microscope. Loosen the screw just enough that the camera or Lucivid rotates in the holder as it is turned by hand, but not so loose that it spins freely. If you are adjusting Lucivid, hold the Lucivid tube carefully so that it does not move in or out, as this changes the focus of the Lucivid it may alter calibration settings.
7. Start with the object at one end of the field of view, just touching one of the grid lines. Move the object all the way to the other side of the field of view. The object moves away from the grid line if your alignment needs to be corrected. While looking at the specimen on the monitor or through the eyepieces (if using Lucivid) gently turn the camera or Lucivid so that you move the object about half way back to the grid line from its stopping position. Now move the stage in the Y-axis so the object is once again just touching one of the lines, and move back and forth in the X-axis to check alignment. Repeat this procedure until the object tracks perfectly along the horizontal grid line.
8. Tighten the setscrew and recheck the alignment. Often the act of tightening the screw alters the alignment slightly, so it may take a few tries to get perfect alignment. Try tightening the setscrew part way, making final adjustments, and then tightening the rest of the way.
9. Repeat steps 6-8 until alignment is perfect.

To ensure the best alignment, start with a high power lens, and then recheck with a low power lens. This checks the alignment over a greater path of X-axis movement.

Parcentric And Parfocal Calibration

You perform parcentric and parfocal calibration to account for parfocal (focal plan) deviations and parcentric (imperfect collimation) differences among different objectives. Parfocal differences are associated with lens design and mounting. Parcentric differences are associated with the mounting of the lens in the nosepiece.

Most lenses—even those in a matching set—are not perfectly parcentric or parfocal. You should check and adjust the parcentric and parfocal calibration whenever you remove lenses from the nosepiece and then reinstall in other positions.

More About Parcentric And Parfocal Calibration : *Parcentric calibration* makes it possible for Stereo Investigator to shift the tracing in the XY-plane automatically when a new lens is selected to compensate for the parcentric differences in objectives. The tracing moves to line up with the new specimen position, but the stage does not move in the XY-plane. This means that if, for example, an object is traced with a low power objective and then you switch to a higher power objective, the object and tracing are still aligned when viewed through the new lens.
Parfocal calibration allows Stereo Investigator to automatically move the stage in the Z-axis to compensate for differences in focal lengths of lenses. With a proper parfocal adjustment, an object that is in focus with one objective lens is also in focus after the next objective lens is selected. It is important to note that this works much better when moving from high power objectives to low power, as the focal depth is much smaller for a high power lens. When changing from a low power to a higher power, realize that the parfocal adjustment may not put your specimen in perfect focus, but it should be close.

In order for these calibrations to be used by Stereo Investigator click **Options>General Preferences** and select the **Lens** tab. Check the **Enable Parcentric** and **Enable Parfocal** checkboxes.

Performing Parcentric And Parfocal Calibration

Before starting, be sure that all lenses are firmly screwed into the nosepiece, and that they have all been properly calibrated. Also check the alignment of the camera or Lucivid. Once the Parcentric/Parfocal calibration has been performed, continued accuracy is dependent on the lenses staying tightly screwed in and in the same turret positions. If you remove lenses for any reason, it is recommended to redo the calibration before resuming work. In addition, if you place lenses in different turret positions, the parcentric and parfocal calibrations are no longer accurate due to the minute differences in position of the lens holders on the turret.

1. Start by finding a slide containing a clearly identifiable point-like object, such as a cell or piece of dust, that is visible with all lenses. You should also make sure that your motorized stage and focus are enabled.

   Use a corner of the smaller calibration grid for this calibration. At high magnification, extend the arms of the cursor to line up with the edges of the box rather than clicking on the "corner", which at high power is quite rounded.

2. Find the object of choice, and center it in the tracing window at the highest magnification used. Once the Parcentric/Parfocal series is started, the movement arrows can be used to move the stage if the object leaves the field of view, but Joy Free is not available.
3. Click **Tools>Parcentric/Parfocal Calibration**. The Parcentric/Parfocal Calibration dialog box is displayed.
4. Select a lens type and click **OK.**

5. In the **Select Lenses for Calibration** select lenses that you do not plan to use or that are no longer on the turret and click **Discard Lenses from Calibration List**. Be sure that all lenses in the calibration list are actually on the microscope and have been calibrated. **insert graphic**To move a lens to the end of the left hand list use the discard button to remove the lens. Next, use the replace button to move the lens back to the left side list. The default order of the lenses is from highest magnification to lowest magnification. This is the preferred lens order for parcentric and parfocal calibration. Click **OK** when all appropriate lenses have been selected.
6. A dialog box asks for the first lens in the list to be used. Rotate the turret on the microscope until the lens snaps into place.
7. Carefully focus on a point-like object on the slide, and click on the point. The calibration procedure prompts for the next lens in the list to be used. Follow the on-screen instructions. Instructions are given to rotate the turret to each lens in turn and to focus on the chosen point and click on it before moving on to the next lens. The lenses are added in order from highest magnification to lowest. This order is used to ensure that the object is visible in the field of view for all lenses. Focus only with the knob on the joystick or with the Fast Focus buttons if your microscope has an external Z focus controller and does not have a focus encoder. If your system has a focus encoder or internal Z motor, you can use the course or fine focus knobs to focus.

Remember that focusing down through tissue brings the stage closer to the objective lens. Do not use the fast focus in the downward direction if the slide is already very close to the lens.

8. Once the calibration is complete, Stereo Investigator asks if you want to enable or disable Parcentric/Parfocal at this time. If these options are enabled, every time a new lens is changed in the Lens box, the tracing moves in the XY-plane to match the new specimen location, and the stage moves in the Z-direction to bring the specimen into focus.

If the specimen has moved out of the current field of view as seen through the new lens, use the **Macro View** window or **Go To** to move to the location of the active tracing. **Center Last Point** is a convenient way to return to where you left off tracing after changing lenses.

Changing Parcentric And Parfocal Calibration

If you need to change either or both calibrations, click **Tools>Parcentric/Parfocal Calibration** and then click **Edit**. The **Parcentric/Parfocal Fine Tuning** dialog box lets you edit the X, Y and Z values; however, the lens name, type, and screen resolution cannot be edited. Click on a value to edit it.

Focus (Z-Step) Calibration

This procedure only applies if your stage controller is equipped for Z-axis position control, i.e. if you have a motorized Z-axis and/or a Z-axis position encoder or a Z-axis transducer. If you do not have a motorized Z-axis or position encoder, refer to the instructions in Focus Step Size Calibration.

Perform the following steps after starting Stereo Investigator with the stage controller enabled:

If the microscope fine focus knob has micron markings, set these to their zero position. Make sure that the units of this scale are in microns of stage movement; some microscopes use each mark to represent two microns.

Select an Oil lens, or select **Tools>Edit Lens** and temporarily change the lens type of the current lens to Oil.

Select **Move>Set Stage** Z and set the Z position to 0.0.

Focus down (move the stage upwards) 10µm. Users with a motorized Z-axis without a Z encoder should use the focus knob on the joystick to do this.

Check that Stereo Investigators correctly reporting a depth value of close to -10.0µm. (The third value in the left portion of the status bar at the bottom of the tracing window should read -10.0).

If the depth calibration is incorrect, select **Options>Stage Setup** and correct the value of the Z Step Size field. If the Z value reported was -20 instead of -10, you would change the Z step size to 1/2 the current value. If the Z value reported was 10 instead of -10, change the sign of the Z step size.

The Z step size is normally a decimal number representing a relatively simple fraction. The most common Z step sizes are 0.01 and 0.02 if you are not using a focus position encoder. If you have a focus position encoder, settings of 0.1, 0.25, and 0.5 are common.

If you modified a lens type to Oil, don't forget to change it back to its original type.

Once the focus calibration has been performed, the calibration can be verified by measuring the thickness of a known object, such as a coverslip. Do this by drawing 2 lines on a coverslip, a horizontal line on one side, and a vertical line on the other. By focusing at the intersection of the lines, and moving the focus from the horizontal line to the vertical one, the thickness of the slide as measured by Stereo Investigator can be compared to the actual thickness, as reported by the manufacturer or measured with calipers.

Calibrating the focus step size

If your Stereo Investigator system does not have a focus position encoder or internal Z motor with encoder, the fixed Z step size can be found in the following table. These step sizes are fixed, based on the internal calibration of the Z motor or encoder, and should not need further calibration.

Common Step Sizes

STAGE	ENCODER SETUP	AXIS (-ES)	STEP SIZE
Prior	MT12-, direct	Z	+/- 0.1
Ludl	MT12-, direct	Z	+/- 0.05
ANY	MT12- via EXE610	Z	0.25
ANY	MT12- via EXE650	Z	0.1
ANY	MT12- to ND281	Z	NA
Ludl	none (Z motor only)	Z	0.01 or 0.02
Märzhauser	none (Z motor only)	Z	0.005
ASI	none (Z motor only)	Z	0.02
Prior	none (Z motor only)	Z	0.1
MICROSCOPE	**MODEL**	**AXIS (-ES)**	**STEP SIZE**
Zeiss	Axioplan 2	internal Z	0.025
Zeiss	Axioplan 2	internal Z	0.05

Zeiss	Axioskop 2	internal Z	0.05
Leica	DMRE	internal Z	0.01

To check the calibration if your system has a focus position encoder or internal Z motor, see Focus (Z-axis) Calibration on page 26.

Setting up a Z Step Calibration

If you have a system without an encoder, you can determine your Z step size using the Focus Step Size Calibration. After selecting Tools>Focus Step Size Calibration, you are presented with a dialog box asking if your microscope has a means of determining how much the stage has been moved.

- If your microscope has labeled tick marks on the focus knob showing the amount of stage movement, click **Yes**, and follow the instructions in the dialog boxes for setting the Focus Step Size Calibration.
- If your microscope does not show the amount of focus knob movement, click **No**, and follow the instructions for measuring an object of known thickness to determine your Z step size.

Be sure that you are using a high power lens with a high numerical aperture, to get the smallest focal depth possible.

In order to do this calibration, you need an object of known thickness on which you can make marks indicating the top and bottom. We recommend using a glass slide or coverslip for this calibration (if you are using a coverslip, you can place it on top of a slide). In order to clearly focus at the top and bottom, we have found the easiest method is to use a marker to make 2 perpendicular lines, one on top and one on the bottom.

Using the Results of a Z Step Calibration

The results of the calibration are presented in a dialog box at the end of the Z Step Calibration procedure, but are not automatically entered into Stereo Investigator. The results of the Z Step Calibration are likely to be a little bit off the actual step size, due to the subjective nature of focusing. However, it should give you a number very close to the correct step size, which should be similar to one of the numbers found on the table at the beginning of this section.

The Z step size is normally a decimal number representing a relatively simple fraction. The most common Z step sizes are 0.01 and 0.02 if you are not using a focus position encoder. If you have a focus position encoder, settings of 0.1, 0.25, and 0.5 are common.

Enter the correct step size into the appropriate location found under Options>Stage Setup. If the stage seems to move in the opposite direction of what you expect, try changing the sign (+ or -) of the step size. For more help with setting the correct step size, please contact MBF Bioscience directly.

Grid Tune Current Lens

This option provides a very efficient method for lens calibration.

To fine-tune the calibration settings for a lens use the following technique:

1. Place the MicroBrightField, Inc. graticule slide on the microscope and bring either the large or small graticule squares into focus.

On most systems it is convenient to view the larger graticule squares when using objectives up to 10X and the smaller graticule squares with objectives more powerful than 10X.

2. Select **Tools>Grid Tune Current Lens**. The Grid Tune Current Lens dialog box is then presented.
3. The name of the currently selected lens is displayed in the Name field. Make sure this matches the objective lens in use on the microscope.
4. In the **Box Size** field, type in the size of a single graticule square visible on the screen or in the eyepieces. The MicroBrightField, Inc. graticule slide has squares that are 25µm and 250µm.
5. Click **OK**. Dashed white lines, which comprise the grid tuning pattern, appear on the screen.
6. Place the cursor over the anchor symbol (found at the intersection of a horizontal and a vertical dashed line). The anchor disappears when the cursor is directly over it.
7. Hold down the left mouse button and drag the anchor's intersection to an intersection of two black lines on the graticule slide that is located near an edge of the screen. Position it carefully so that this vertex exactly matches this corresponding vertex of the graticule slide.

   Placing the first intersection near an edge of the screen maximizes the distance from the anchor along a given axis. A small error in one square is magnified over larger distances, so it is best to perform the calibration over as great a distance as possible. However, your image may show some optical aberrations near the edges, so be sure to only calibrate in the uniform region of the image.

8. Examine all the vertical dashed lines in relation to the vertical black lines of the graticule slide. If some of the dashed lines do not lie exactly on the black lines, place the cursor on a vertical dashed line (using a dashed line farthest from the anchor works best); the cursor changes into a double arrow, pointing both left and right. This indicates the cursor is directly over the dashed line. Now hold down the left mouse button and drag this vertical dashed line to the left or right until it lies exactly on top of its corresponding black line on the graticule slide.
9. Make sure that all the dashed vertical lines lie directly on top of their corresponding black vertical graticule lines. If not, readjust them using this same technique.
10. Perform the same type of adjustment for the horizontal dashed lines relative to the black horizontal lines of the graticule slide.
11. When all the dashed lines (or at least those in the center third of the field-of-view) lie directly on top of their corresponding black lines on the graticule slide, the calibration settings for this lens have been properly fine-tuned.
12. With the cursor in the tracing window, click on the right mouse button to bring up the context sensitive right click menu. Move the cursor over the **Adjust Current Lens With Grid Tuning** entry and click on it with the left mouse button to complete the grid tuning procedure.

Chapter

5

Moving Around in Stereo Investigator

Before beginning a project in Stereo Investigator, it is essential to understand the ways in which Stereo Investigator communicates with your stage controller and to understand how various commands allow you to control this stage movement.

The topics that follow contain many important concepts that should be reviewed by all new users before beginning to Stereo Investigator.

The most important concept is that of registration of the tracing and the stage. *Registration* means that the tracing and specimen are in correct and proper alignment, even when you move the stage.

Most of the Move commands retain that registration. That is, when the stage moves to a new location, the tracing moves as well to remain perfectly aligned with the specimen on the slide. There are times, however, when you want to move the stage independent of the tracing, for example, when moving to a new section. The Joy Free and Align commands allow for this separation of stage and tracing, as do several of the commands on the Tools menu.

The best way to familiarize yourself with these features is to put a specimen under the microscope, do a few quick tracings and then try these features to see what happens with the tracing and specimen.

Using The Joystick

The Joy Free and Joy Track commands let you perform free movement with the joystick. The joystick is disabled unless one of these commands is selected. The essential difference between these two commands is that Joy Free allows free movement with the joystick that is not tracked by Stereo Investigator. Common uses for **Joy Free** include moving the stage to a new section on a slide, placing a new slide, or aligning new sections with previous tracings. **Joy Track** also lets you move the stage with the joystick, but Stereo Investigator tracks those movements and realigns the specimen and tracing once the move is complete.

An easy way to remember the difference is that Joy Track keeps track of where it has moved.

Remember that **Joy Track** keeps track of movements in X, Y and Z. To focus without changing the Z position of the tracing, remember to first activate **Joy Free.**

If movement has been performed using **Joy Free** and you find that you want to return to the original alignment of specimen and stage, right-click and choose **Switch to Joy Track**. Alternately, if you are in **Joy Track** mode, you can right-click and choose **Switch to Joy Free**.

Once you exit **Joy Free**—either by clicking the **Joy Free** button, or by right clicking and choosing End Joystick Mode—it is not possible to return to the previous alignment of overlay and specimen.

When in **Joy Free** or **Joy Track** mode, all tracing and mapping functions are disabled. You must exit Joystick mode before returning to tracing.

To Start Joy Free or Joy Track

- Click **Move>Joy Track** or **Move>Joy Free**.
  -or-
  During tracing, right-click in the tracing window and choose **Joy Track** or **Joy Free**.
  -or-
  Click the **Joy Free** toolbar button

To exit Joy Free or Joy Track

- Right-click in the tracing window. There are two choices in the right click menu that both end the joystick mode.
  - To finish in the mode originally selected, choose **End Joystick Mode**.
    -or-
    Choose **Switch To** and the joystick mode ends in the new mode listed.

Aligning The Tracing And Specimen

If you are not doing a serial section reconstruction, the most common reason that the tracing and specimen are out of alignment is that you have moved the slide or switched lenses (if not using Parfocal/Parcentric, or if that calibration is off a little bit). It could also be that a simple mistake has been made.

Some of the methods for aligning sections move only the current section into alignment; others move the entire stack of images in a Serial Section Reconstruction. Pay close attention to the notes following the description of each alignment method; these tell you whether the method moves all sections or only the visible ones!

If you are returning to a section previously started and you have taken the slide off the microscope, you also need to align the tracing and specimen . Outlined below are the basic methods for lining up a tracing and specimen.

If you have been tracing with the wrong lens that you selected from the **Lens Selection** List box, there is a loss of registration between tracing and specimen that cannot be re-aligned easily, as the size of the tracing does not match the size of your specimen. Try using **Tools>Shrinkage Correction** to scale the tracing to the correct size (once the correct lens has been selected). If you are able to get the tracing to the correct size, you can then use the methods described here to align it with the tissue at the correct magnification.

- **Move>Joy Free**: Use Joy Free to do an initial alignment of tracing and section when the slide has been moved, or if the alignment is off by a great deal. Then the final alignment can be performed with one of the tools described below. Select Joy Free and then move the tracing until it is closely lined up with the specimen. Remember to deselect Joy Free before continuing the rest of the alignment. Not all of the tools below need to be used for every alignment; usually a combination of operations gives best results.

  Joy Free moves all sections whether displayed as visible or hidden.

Joy Track moves the stage and the data file together, and thus is not used for aligning the stage and tracing.

- **Tools>Match:** Match provides a best fit between the tracing and new specimen based on the location of two to 99 points. This is the easiest method to obtain a quick, good fit between the image and the tracing. **Match** rotates and moves the overlay, without changing it, to get the best match with the specimen. To perform a **Match**, specify the number of pairs you are going to use for matching the tracing with the tissue. For each pair, first pick a point on the overlay and then pick the corresponding point on the image. Repeat this for subsequent pairs. If this requires moving the stage, use **Move>Joy Track** or the arrow buttons on the status bar to find the next pair of points.

Match moves all sections whether displayed as visible or hidden.

- **Move>Align Tracing:** This operation is also accessed through a right click in the tracing window. Align Tracing moves the tracing in the X, Y and/or Z-axis, but does not rotate the tracing. When this option is selected, instructions appear in the status bar prompting you to first pick a point on the overlay (tracing) and then to choose the point on the specimen where you want this point to appear. Refocus if necessary before clicking on the second point to change the Z position of the tracing.

Align Tracing moves all sections whether displayed as visible or hidden.

- **Tools>Rotate Tracing** allows for simple rotation around the reference point. This tool only rotates visible sections. To align a new section in a serial section reconstruction, be sure that all sections are visible (Options>Display Preferences>View Tab, deselect **Show current section only**). Rotate Tracing has the advantage that the movement of the tracing can be seen while the adjustment is being made.

Rotate Tracing moves only sections displayed as visible.

Moving Imported Images

Both **Joy Free** and **Joy Track** are disabled when you work with imported images. Instead, you use the **Move Image** commands.

Move Image is similar to **Joy Free** in that it moves the imported or acquired image without moving the tracing. This is useful for lining up a new image with a previous tracing when using acquired images for a serial section reconstruction.

Move Image and Tracing is similar to **Joy Track** in that it moves the image while maintaining the registration between the image and tracing. **Joy Track** keeps track of stage movement in order to realign the tracing with the moved specimen. **Move Image and Tracing** does not move the stage, but moves the tracing and image simultaneously.

In addition to dragging the images it is possible to nudge images with the arrow buttons on the keyboard while in **Move Image** mode. Each click of an arrow button moves the images 1 pixel on the screen. Hold CTRL down while pressing the arrow keys to move the images 10 pixels at a time.

Multiple Images

Using the Spatially Organized Framework for Imaging (SOFI) technology, multiple images may be positioned in 3D space. This allows data acquisition of specimens larger than a single field-of-view. The images do not need to be overlapping.

Working With AutoMove

AutoMove is an automatic centering procedure for use with motorized stages. It acts in conjunction with a dashed rectangular boundary, known as the **AutoMove Area** to allow for the continuous tracing of structures larger than a single field-of-view . Click **Move>AutoMove** to activate it, or use the **AutoMove** button.

Open the **Macro View** window to get a better view of the **AutoMove** area. You'll be able to see a reduced view of the entire area.

When active, **AutoMove** automatically centers the display when you click outside **AutoMove** window area. Both the tracing and the stage move in unison so that there is no loss in registration. Note that there may be a momentary delay as the stage moves to its new location. Continue tracing uninterrupted.

If the **AutoMove** area is defined "backwards", that is, by clicking the lower right corner first, then the upper left, each point that is drawn is centered immediately, whether it is inside or outside the AutoMove area.

AutoMove Settings

Set the AutoMove Area boundary to encompass the central two thirds of the screen. This reduces visual confusion when your stage executes a move to center a peripheral point.

To define the AutoMove Window

1. Click **Move>Define AutoMove Area**.
2. Click and drag from the upper-left to the lower right to define the **AutoMove** area. If you drag from lower-right to upper-left, each drawn point is immediately centered, whether inside or outside the **AutoMove** area.

Center Point

You can define the **Center Point** as either the center of the tracing window or the center of the **AutoMove** window. These are not necessarily the same, depending on where you place the **AutoMove** window. The center of the tracing window is the default. To center the point at the center of the **AutoMove** window, Click Options>General Preferences>Movement tab and check **Center Cursor in AutoMove Area**.

Working With Meander Scan

Meander Scan is an automated scanning procedure that is used to ensure that all points within a closed contour are viewed by moving systematically through the contour. The directions that follow walk you through the steps for setting up and executing a Meander Scan.

To perform a meander scan

1. Draw a closed contour around the region of interest.

   Use a low-powered lens to draw the contour. If your region of interest has contours within contours, Meander Scan treats the interior contours as an exclusion zone, and does not visit these. If you want to include these areas, select these interior contours, right-click and choose Hide Selected Contours.

2. Click **Options>General Preferences>Movement** tab. Set the **Field Movement** size to 75% of Screen Size, and click **OK**.
3. Click **Move>Meander Scan**. In the **Meander Scan** dialog box, click **Start Meander Scan**. If there is more than one contour, Stereo Investigator displays the **Macro View** window. Click inside the desired contour to scan.
4. Mark, trace, or map anything within the current field-of-view.
5. To move to the next scan site, click **Move>Meander Scan** and select **Next Scan Site**.
   -or-
   Right click in the tracing window and choose **Next Scan Site**.
   -or-
   Use the **Next Scan Site** button. If you think you missed something in a previous section, click **Previous Scan Site**.
6. Click **Move>AutoMove** if there are structures that extend beyond one field-of-view.

   If using AutoMove, we recommended that you return to the previous site before continuing mapping, just to be sure nothing was missed before AutoMove took you away from that scan site.

7. When you are viewing the last site, clicking **Move** ends **Meander Scan**.

Chapter 6

Contours and Tracing

Tracing Contours

Most of your work in Stereo Investigator involves contour mapping, and the use of contours is necessary for many of the analyses in Stereo Investigator. Take the time to get comfortable with tracing contours when you are first getting started with Stereo Investigator.

How Do I Trace Contours?

The instructions below assume that Stereo Investigator has been started, a specimen is in view, and a reference point has been placed.

1. Click **Trace>Contour Mapping**.
2. Choose a contour from the **Contour** box.

   To change the name and/or color of a contour, click **Options>Display Preferences>Contour** tab.. Contours can be renamed and new colors chosen by following the directions on the left side of this dialog box.

3. Select your tracing method. Right click in the tracing window, and choose **Simple Click Tracing**, **Rubber Line Tracing**, or **Continuous Tracing**. The method chosen is strictly a matter of preference.

   Many users prefer **Simple Click Tracing**, in which you click at each new point in the contour and the last point is connected to the previous one by a straight line. **Rubber Line Tracing** works similarly, except that you can see the line as it extends from the last point to your new location. **Continuous Tracing** allows you to hold down the left mouse button and "draw" around the contour as if the mouse controlled movements of a pen**. Continuous Tracing** is not recommended if focal depth is important as it is easy to forget to stop tracing and refocus. The tracing method can be changed at any time during the tracing by right clicking in the tracing window.

4. Trace the contour of choice.
5. If the contour is larger than the field-of-view, it is easiest to trace if **Move>AutoMove** is turned on.

If you make a mistake, click **Edit>Undo** to erase the last drawn points one by one back to the beginning of the contour

Focusing while tracing: If the system is configured with a focus meter, control the focus via the use of the joystick or the fast focus tool.
When using a microscope with a built-in Z motor system, focus using the microscope's focus knobs

and the focal depth changes are reported to Stereo Investigator.
If your system has stage transducers (encoders), focus manually and Stereo Investigator offers prompts to move the stage to the required positions as needed. The transducer's position readout display guides you during positioning.

What Are Open And Closed Contours?

Contours can be either open or closed. A contour must be closed to obtain measurements of the area within the contour. An open contour can be used to measure the length of a curved line.

To End a Contour

- Right click in the tracing window and choose either **End Open Contour** or **Close Contour**.

An open contour can later be closed or appended by editing.

Automatic Contouring

Automatic Contouring lets you interactively trace live or acquired images, an often time-consuming and tedious task. Based on the parameters and options you set, Automatic Contouring will trace a contour and also move the stage to follow the image's contour.

Although Automatic Contouring is a powerful and intelligent command, you will still need to examine the traced contour and perform some editing on the results.

Starting An Automatic Contour

Automatic Contouring works on live and acquired images.

To use automatic contouring

1. Place a reference point and load an image.
2. Right-click anywhere in the work area and choose **Automatic Contouring**. Stereo Investigator opens the **Automatic Contouring** tools panel. As with any tools panel, you can move it anywhere on your monitor.

3. Start tracing a contour in the desired region—Automatic Contouring requires two points as a minimum to start.
4. Click the **Forward** button to start tracing.

You can let the tracing complete and then edit it, or you can delete points and change parameters and options and resume.

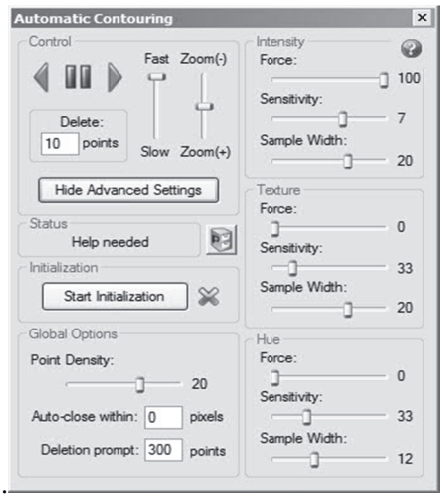
A Note about Zooming: Automatic Contouring works on an image **exactly** as it is displayed. For that reason, the zoom level has an effect on automatic contouring accuracy. A zoomed-in image displayed more and finer detail, and the tracing will reflect this. You will need to experiment to determine which zoom level is best for your study.

To delete points

- Click **Back** to delete the set number of points. You can click to delete points all the way back to the start of Automatic Contouring.
  -or-
- Right-click on the point you want to be the last point (the point you want to start tracing from) and choose **Delete Points to Here**.

Advanced Settings

Advanced settings contain options and parameters that affect the automatic contouring results. Click the **Advanced Settings** button to work with these settings



Global Options

Point Density controls the frequency of the point placement during tracing. A lower setting here means fewer points placed.

Auto-close within pixels closes the current contour as soon as an added point comes within the set number of pixels of the contour start. 0 turns disables this feature. We recommend starting with a value of 30.

Deletion prompt controls a message box that Stereo Investigator displays if more than the value set here number of points are to be deleted. This helps protect you against accidental deletion of a large contour.

Intensity, Texture, and Hue

These control three different available methods for automatic contouring. Each, **Intensity, Texture,** and **Hue,** have three controls: **Force, Sensitivity,** and **Sample Width**.

Force controls the amount of force that a particular method has on each point placed. A smaller **Force** value tends to move on more of a straight line.

Sensitivity controls when automatic contouring needs to stop and ask you for help—you will need to examine the tracing and make some decisions. A lower **Sensitivity** value requires less interaction, but it is more likely to wander into undesirable image areas.

The **Sample Width** value defines a region on either side of the contour which is sampled during contouring for making point-placement decisions. A larger **Sample Width** samples farther away from the contour.

Which Control Is Best to Use?

It is possible to use multiple methods simultaneously. However, you may get the best results by using one method at a time. To disable a method, set its forces to 0.
Again, we can't give you a hard and fast setting for each and every situation. Experimentation with your data will yield the optimum settings for your study.

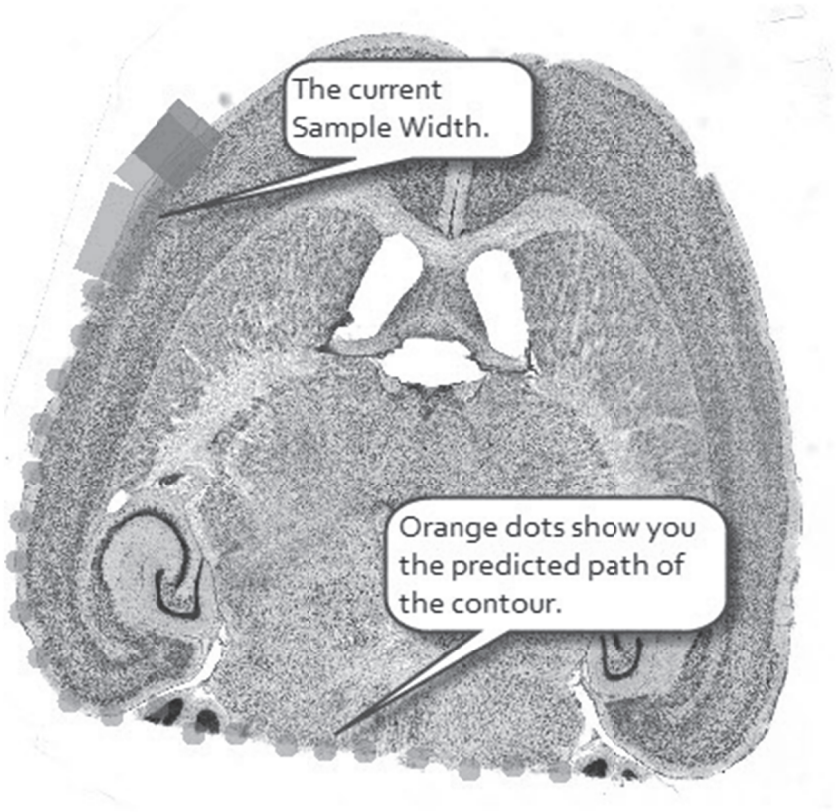
Using presets

We've included a set of preset option settings for you to use as starting points to creating your own settings. Try out these presets—they'll give you a sense of how the different parameters and options affect the results.

To use the presets, click the Presets button and choose a preset from the list.

Determining Best Settings

The best method for determining correct settings to use is **Preview** mode, which lets you make changes to settings while tracing. Begin placing a few points. Stereo Investigator displays blue semi-transparent boxes (in gray here) to represent the current Sample Width and orange circles (gray here) show the predicted path using the current settings.



You can make adjustments to the settings and see them reflected on screen. To leave **Preview** mode , click **Stop Initialization** or start Automatic Contouring.

Contour Measurements

You can see contour measurements as you draw contours with the **Contour Measurement** tool panel, available from the **Options>Display Preferences>Accessories** tab menu, or by clicking the **Contour Measurements** button. This tool panel provides a full, printable analysis of all contours visible in your current tracing. You can immediately see the analysis of each new contour as it is drawn.

Basic Contour Information

Contour Name

This is the user-defined name given to the contour. To change names, click **Options>Display Preferences>Contours** tab.

Area

The 2-dimensional cross-sectional area contained within the boundary of a closed contour. The area is the profile area. The contour is considered to be flat when the calculations are computed, thus giving a 2-dimensional result. The 3-dimensional quantity is called surface area. The area can be displayed in square microns, square millimeters, or square centimeters. Use **Options>General Preferences>Numerical Formatting** to select the desired units in which to display these results. Area is not defined for open contours.

Perimeter

The length of the contour for either open or closed contours. Unlike area, the length does take the Z positions of the coordinates into account. The perimeter can be displayed in square microns, square millimeters, or square centimeters. Use **Options>General Preferences>Numerical Formatting** to select the desired units in which to display these results.

Perimeter is a tool that you can use to measure a two-dimensional distance that is larger than a single field-of-view. If you have two points and want to determine the distance between them, choose **Contour Mapping** mode, click once on the first point and then use the arrow movement buttons, **Joy Track**, **Go To** to move to the second point. Click on the second point, and a line connects the two points. Right click and select **End Open Contour.** Display the **Contour Measurements** window and the perimeter of the contour you just drew is the two-dimensional distance between those two points. Another method for measuring the distance between 2 points, even across multiple fields of view, is the **Quick Measure Line** tool. The tool works the same as drawing a 2-point contour except no contour is drawn and the results are reported immediately.

Object Markers

Gives a summary of the total number of markers attached to a given contour. Markers are automatically attached to a contour if they are placed while the contour is being drawn (before **End Open Contour** or **Close Contour** is selected). If markers are drawn before the contour is started or after the contour is completed, the markers can be attached by the user within the Editing Mode.

Luminance Information

Enable **Collect Luminance** before you trace a contour. This feature is only available on acquired images.

To collect luminance information

- Click **Collect Luminance** button or **Imaging>Collect Luminance Information**.

To collect the luminance information after a contour has been drawn

1. Click **Collect Luminance** button or **Imaging>Collect Luminance Information**.
2. Right-click and choose **Redo Luminance,** and then left click within the contour of interest. The luminance information is automatically collected. Luminance information is only collected from one contour at a time when using **Redo Luminance**, so you need to go through the process of selecting **Redo Luminance** and clicking inside each contour of interest.

Displaying Luminance Information

Double click on any of the luminance values the available luminance information for that contour. If you want to see a thumbnail of the image and a histogram of the luminance values within that contour, use **Options>General Preferences>Luminance** tab to enable **Save Image Histogram and Save Image**. The image of the contour and the luminance histogram can both be copied to the clipboard and pasted into other programs for further analysis.

Click on any of the column headings to re-order the listings based on that parameter. Click a second time to reverse the order.

Brightness

This column displays the average luminance of pixels inside a closed contour. Luminance has a range from 0 to 255 for each pixel. A black pixel has a luminance of 0, while a white pixel has a luminance of 255. For color pixels, the luminance is defined as:
(.299*Red)+(.579*Green)+(.114*Blue)

StdDev

This column displays the standard deviation of the luminance of the pixels inside the contour. This gives a numerical description of the distribution of collected luminance values.

Min

This column displays the minimum luminance of the pixels inside the contour.

Max

This column displays the maximum luminance of the pixels inside the contour.

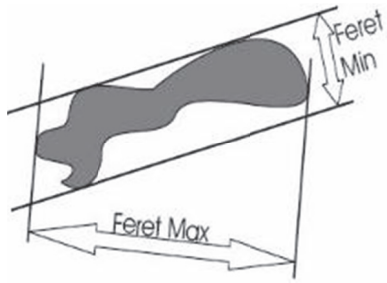
Total

This column displays the total number of pixels inside the contour at this lens magnification.

Shape Information

Feret Min (mm) and Feret Max (mm)

These two columns display the largest and smallest dimensions of the contour as if a caliper was used to measure across the contour. The figure shown here illustrates this concept.



Note that the two measurements are independent of one another and not necessarily at right angles to each other.

Aspect Ratio

The degree of flatness of a contour shape as the ratio of its minimum diameter to its maximum diameter. Range of values is 0-1. A smaller aspect ratio indicates a flatter contour, while an aspect ratio approaching 1 indicates a rounder contour. A circle has an aspect ratio of 1. Remember that aspect ratio describes the 2-dimensional contour, and may not describe the 3-dimensional shape of particles being observed.

Compactness

Describes the relationship between the area and the maximum diameter. The range of values is 0 to 1. A shape with compactness approaching 1 has a large area in relationship to its perimeter, with a circle being the most compact shape (compactness for a circle = 1). A square has a compactness of 0.8.

Shape Factor

Gives information about the complexity of a contour as defined by the relationship between the perimeter and the area. A contour with a large shape factor has a large perimeter as compared to its area, indicating a convoluted outline. A small shape factor indicates a small perimeter as compared to area. A circle has the smallest shape factor, with a value of approximately 3.54).

Form Factor

As the contour shape approaches that of a perfect circle this value approaches a maximum of 1.0. As the contour shape flattens out, this value approaches 0. The form factor differs from the compactness by considering the complexity of the perimeter of the object. For example, a circle with a smooth perimeter has a compactness of 1 and a form factor of 1. If the smooth perimeter is replaced with a finely jagged edge (like a cell covered in microvilli), the compactness is still near 1, but the form factor is much smaller since the perimeter is lengthened considerably.

Roundness

Roundness is closely related to compactness. Roundness is the square of the compactness. Just as compactness ranges from 0 to 1, roundness has the same range. By squaring the value, it is easier to differentiate objects that have small compactness values.

Convexity

One measure of the profile complexity is convexity. Convexity is calculated as (Convex Perimeter/Perimeter). A completely convex object does not have indentations, and has a convexity value of 1. Therefore, circles, ellipses, and squares have convexity 1. Concave objects have convexity values that are less than 1. Contours with low convexity have a large boundary between what is inside and what is outside for their size.

Solidity

The calculation of solidity is based on the concept of a contour's convex area. The area enclosed by a ‘rubber band’ stretched around a contour is called the convex area. A circle, square or ellipse has a solidity of 1. Indentations in the contour take area away from the convex area, decreasing the actual area within the contour. Solidity is the area of the contour divided by the convex area. Notice that it is possible to have contours with low convexity and high solidity and vice versa.

Error Coefficients

Area Error Coefficient

This value can be used to determine an upper bound for the error of the calculation of a contour area, assuming the contour traces the region of interest as well as possible at a given lens magnification. The actual area within a contour that is a single pixel thick is the area shown in the contour measurements data +/- [(Area Error Coefficient)(Pixel size in microns)]. If the tracing was not drawn perfectly, but never varied by more than 5µm from the boundary of the region of interest, the actual area is the area shown by Stereo Investigator +/- [(5)(Area Error Coefficient)].

The basic concept behind the area error coefficient can be imagined as drawing the perimeter of an object with a thick pen. The wide line covers an area around the object. The true boundary of the object lies within the wide line. The outside edge of the wide line is the largest area enclosed by the contour; presumably larger than the actual area of the object. The inside edge encloses the smallest area enclosed by the contour; presumably smaller than the actual area of the object. The area represented by the wide line

represents the possible error in the area of the object – this error is maximized if either the inside or outside edge traces the boundary of the object perfectly. The calculation given above estimates the area of the wide line (which may not be all that wide, but is at minimum one pixel in width). In general, the calculation actually provides a value that is larger than the actual error. That is why we say that the value is an upper bound.

Perimeter Error Coefficient

This value can be used to determine an upper bound for the error of the calculation of a contour perimeter; assuming the contour traces the region of interest as well as possible at a given lens magnification. The actual perimeter of a contour that is a single pixel thick is the perimeter shown by Stereo Investigator +/- [(Perimeter Error Coefficient)(pixel size in microns)]. If the tracing was not drawn perfectly, but never varied by more than 5 microns from the boundary of the region of interest, the actual perimeter is the perimeter shown by Stereo Investigator +/- [(5)(Perimeter Error Coefficient)].

The perimeter error is harder to calculate. Part of the mathematics in the derivation assumes that the contour traced is roughly parallel to the true perimeter. A simple way to think about parallelism is to consider the angle between the true contour and the traced contour. An angle of 0 means that the two are parallel. An angle of 90 degrees means that the two are perpendicular. The derivation of the formula requires that the cosine of the angle is nearly 1. The cosine of 0 degrees is 1. The cosine of 10 degrees is .98. This means that even if the lines are 10 degrees apart a relatively good estimate of length is possible.

Editing Mode

Stereo Investigator provides a separate mode of operation for editing tracings, called the Editing Mode. When in the Editing Mode (as indicated by the depressed **Select Objects** button), you have access to a wide variety of editing options via a right-click. Most of these options can be undone. However, once you exit the Editing Mode, the ability to undo changes is lost. You can't trace or place markers in the Editing Mode, these options are grayed out on the toolbar when the Editing Mode is active. To start tracing or placing markers again, exit the Editing Mode.

Enter And Exit Edit Mode

Use one of the following to enter Edit Mode:

- Click **Select Objects**, on the main toolbar .
- Click **Edit>Select Objects**.
- Click **Edit>Select All Objects**.

Remember: When you are in the Editing Mode, tracing capability is disabled, and the cursor changes to a pointing finger that you use to "point" to the objects you want to edit.

To exit Editing Mode and return to tracing or placing markers:

- Click **Select Objects** again.
- Right click in the tracing window and choose **Exit Selection Tool**.
- Click **Edit>Select All Objects** again.

Selecting And Acting On Objects

Before you can edit an object or objects, you have to select them. This topic explains how to select within Stereo Investigator.

Selecting Objects

You can select a variety of objects, such as neurons, markers, contours, text, or objects by section.

To select an object

1. Click **Edit Select Objects**, **Edit Select All Objects**, or click the **Select Objects** or **Select All Objects** button. Stereo Investigator puts you in Edit Mode
2. To select an object, click on it. Selected objects appear highlighted on the screen. This means that white squares appear to indicate that the object is selected. If text is selected, it appears within a white square. Selected markers appear with a white box over them.
3. To select another object, click on it. Stereo Investigator deselects the currently selected object and selects to new object. the squares disappear from the previously selected object and appear on the newly selected object.
4. To add objects to those already selected, hold down the SHIFT key and click on all the objects to select.
5. Holding down the CTRL key allows you to add or remove objects from a group of selected objects. Clicking on an unselected object adds it to the selected group, clicking on a selected object removes it.

The Selection Box

You can also use the mouse and selection box to select objects.

To select objects with the mouse and selection box

1. To select multiple objects in an area, click at the upper left of the area you would like to outline, hold down the mouse button and move to the lower right of the area of choice, and then release the mouse button. This box can be moved as it is being drawn by holding down the SHIFT key.

   Holding down the SHIFT key while drawing a selection box allows you to add a group of objects to a set of *previously* selected objects.

2. Groups of objects can be deselected, or removed, from a selected group by drawing a rectangle around them starting from the lower right and moving to the upper left. The objects contained within this 'backwards drawn box' are deselected, while the previously selected objects outside the deselection box remain selected.

   This method of deselection can be useful when you are doing multi-object editing.

Acting On Objects

Acting on the objects you've selected is easy.

- Use the **Delete** key or CTRL+Z delete all selected objects.
- Left-click directly over neuron or contour point (within a selected neuron or contour) to turn the cursor into a grabbing hand. Left-click and drag the point to a new location.
- Objects can be operated on once they have been selected. The operations available are accessed by right clicking in the tracing window. In general, right clicking on the object gives you options specific to that object, while right clicking away from any object gives you the more global editing options. For detailed descriptions of the editing operations, See Editing Contours, Editing Markers or Editing Neurons for instructions.
- When you have selected the option to Move an object or objects (as opposed to a single point), the cursor changes to a hand, which closes to a grabbing hand when something is being "held" and moved.

The options that are available for mixed groups of selected objects are somewhat more limited than those available to objects of a single type.

Editing Text: Text is modified much the same as other objects, with the exception that the **Change Text** option lets you change the content of the text, and **Change Font** allows you to choose a new font.

Hidden Objects

Understanding the concept of hidden objects is important for mastery of the Stereo Investigator program. When objects are hidden from view, they are also effectively hidden from the program; editing and most alignment functions do not act on hidden objects.

- To hide objects, select them in the Editing Mode, right click, and choose **Hide Selected** objects.
- To hide all sections other than the one being actively traced, Click **Options>Display Preferences** and check **Show Current Section Only**.

Objects must be restored—not just unhidden—before they can be operated upon within the Editing Mode.

To restore hidden objects

1. Enter the Editing Mode and right click in the tracing window. Select **Reveal Hidden** objects. Revealed objects appear in an olive green color. They are still effectively hidden from the program until they have been restored, at which time they are shown in their original color.
2. Select the revealed objects.
3. Right click and select **Restore Selected objects**.

Don't confuse the Hide Tracing function with hiding objects. Hide Tracing simply turns off the tracing display, and does not actually create hidden objects.

Editing Contours And Points

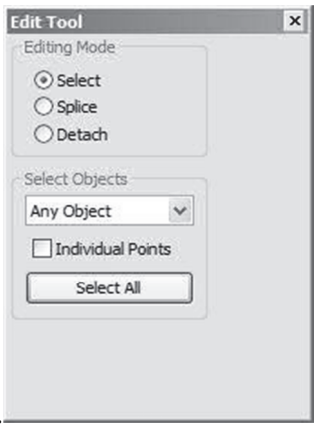
While in Editing mode, you can right-click on a contour in the tracing window to display a list of actions you can perform.

Selecting A Contour Or Contours

You need to select a contour or contours before you can perform an action.

To select a contour or contours

1. Click **Edit Select Objects** or click the **Select Objects** button. Stereo Investigator puts you in Edit Mode and displays the **Edit Tools** panel



2. With **Select** selected under **Editing Mode**, select **Only Contours** from the **Select Objects** drop-down list box.
3. Click on a contour to select it. Stereo Investigator highlights the selected contour.
4. To select another contour, click on it. Stereo Investigator deselects the currently selected contour and selects the new contour.
5. To add contours to those already selected, hold down the SHIFT key and click on the contours to select.
6. Holding down the CTRL key allows you to add or remove contours from a group of selected objects. Clicking on an unselected contour adds it to the selected group, clicking on a selected contour removes it.
7. Click **Select All** to select all contours.

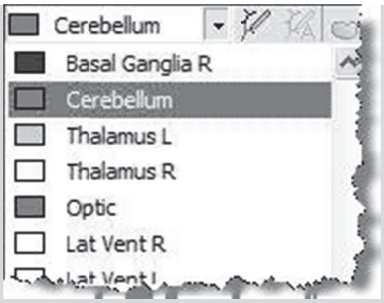
Editing Contours

After selecting a contour or contours, right-click and choose a command from the menu.

- **Change All to Contour Type**: Displays a dialog where you can choose a new contour type.

Contour Type

It is important to understand the concept of Contour Type before editing contour properties. The contour types currently assigned are listed in the Contour box, a portion of which is shown here.



A contour type is defined as a group of contours having the same exact name. These types are changed using **Options>Display Preferences>Contour** tab. When a contour name and/or color is changed this way, all existing contours of that type are changed, the name appearing in the **Contour Box** is changed, and all future placements of that contour type are assigned the new name. This is the correct way to assign names to a contour that is to be used repeatedly in your tracings. If a contour name is changed using the editing mode using **Rename Selected Contour**, only the selected instances of that contour are changed. The name in the **Contour Box** is not changed, and future instances of the contour are not affected.

Exception: If a contour name is changed to exactly match an existing contour name (case sensitive), then the contour is assigned to the new contour type, and all changes to the new contour type made in **Options>Display Preferences>Contour** tab are applied to the edited contour. Assigning the new name does not automatically change the contour color to match other contours of that type, but if the contour color or name is changed in **Display Preferences**, the new properties are applied to all contours with identical names.

- **Move Selected Contour**: Turns the cursor into a hand. Click and drag the contour to a new location.
- **Rotate Selected Contour**: Rotates the contour or group of contours around the center point indicated by a green gunsight icon.
- **Flip Selected Contour**: Displays a dialog which you use to select a horizontal or vertical flip for the contour. Stereo Investigator flips the contour around the center defined as the reference point. If you uncheck **Flip around origin (reference point)**, Stereo Investigator flips two or more contours only, using their center as the origin.
- **Delete Contour**: Deletes all selected contours.
- **Hide Selected Contour**: Hides the selected contours. See Hidden Objects for information on working with hidden objects.
- **Copy Selected Contour**: Copies the selection to the **Clipboard.**
- **Place Contours Into Set**: Displays the **Name of Set** dialog box. Type a name for the set and click **OK**. Stereo Investigator places the contours into a set.
- **Set to Cell Body**: Changes the selected contours to cell bodies. If applied to an open contour, this command closes the contour. If you selected **Fill Cell Bodies** in the **Options>Display Preferences>Neurons** tab, the cell bodies are filled.
- **Append to Selected Contour**: Only available for open contours. This selection exits you from the Editing Mode and returns you to tracing at the last point of the selected contour, indicated by a flashing circular cursor.

- **Add to Start of Contour**: Works like **Append to Selected Contour**, except that it allows you to append to the first point of the contour rather than the last. This selection exits you from the Editing Mode and returns you to tracing at the first point of the selected contour, indicated by a flashing circular cursor.
- **Insert Point in Selected Contour**: Prompts you to click where you would like to place a point.
- **Change Contour Color**: Displays the Color dialog box, which you use to choose an existing color or define a new one.
- **Rename Selected Contour**: Lets you rename the selected contours.
- **Close Selected Contour**: Closes the selected open contour
- **Modify Z Position**: Prompts you to either **Shift Z Values** or **Set Z Values**. **Shift Z Values** allows you to move the contour up or down (by specifying a positive or negative number) a given number of microns. **Set Z Values** allows you to tell the program the correct Z value for that contour. If multiple contours are selected, **Set Z Values** sets them all to the same Z value.
- **Modify Intrinsic Contour Size**: Using the **Modify Contour Size** dialog box, you can modify the intrinsic size of a given contour in three ways: 1) A slider bar allows for dynamic change to the intrinsic size measurement. The value you have selected is shown in **Set**, 2) Use **Set** to specify a new value, or 3) Use the **Scale** to specify a factor by which the intrinsic size should be changed (i.e.: specifying 3 increases the thickness by 3X its current value, a 5mm thick contour becomes 15mm thick). The new value does not dynamically change in **Set** if **Scale** is used, but is changed upon exiting the dialog box.
- **Apply Shrinkage**: This is equivalent to using the **Tools>Shrinkage Correction** command, allowing you to apply shrinkage to a single contour, or to the entire section, if you used **Edit>Select All Objects** to enter the Editing Mode, or if all tracing objects have been selected. **Apply Shrinkage** is most commonly used when flipping sections mounted upside down (Flip a single section). **Apply shrinkage** can be used to correct for known amounts of shrinkage, if you want measurements to reflect the parameters of the tissue before processing. However, this would usually be applied to an entire data stack, rather than to just a few contours.

A Note on Shrinkage Factor: The shrinkage factor acts as a multiplier, and should be the inverse of the actual change in size. If you know that your tissue has shrunk to 25% of its original size, the factor to apply is 1/0.25, or 4. However, if you have a 25% shrinkage, the tissue is now 0.75 of its original size. Therefore, the factor you would apply is 1/0.75, or 1.33.

- **Place Contour in Section:** (Only available if using sections). Places the selected contours into a new section. When this option is selected, Stereo Investigator displays a dialog box which you use to selected the section to place the contour.
- **Fix Z Values**: Corrects large deviations in the Z values of a contour. The Z discrepancy that is fixed by Fix Z Values is the same as the Z value that triggers a **Z Position Out of Range** warning during tracing. (This value can be changed in **Options>General Preferences** under the **Tracing** tab.) When Fix Z Values is selected, any jumps in Z position larger than this specified value are automatically corrected. The Z values shown in the **Orthogonal View** window do not change until you exit the Editing Mode.
- **Operate on Attached Markers**: Applies all editing operations to markers that are attached to the selected contour. Deselect this option to apply the editing operations to the contour and not the attached markers.

- **Detach Markers from Contour**: Lets you bind a contour or set of contours to an object. Later manipulations of the object include the attached contours. A good application of this tool would be to attach boutons that you have marked to their axon. The axon can then be moved, rotated or scaled while keeping the contours in their appropriate locations. To use this tool, first select all contours that you want to attach. After selecting **Attach Contours to Object**, Stereo Investigator prompts you to click on the tree or contour that you would like the contours attached to. Note that this tool attaches contours to a specific branch of a neuronal process, so click *directly* on the appropriate segment.
- **Undo Last Type Change**: Undoes the last change to a contour.
- **Exit Selection Tool**: Returns you to the previous mode.

Editing Markers

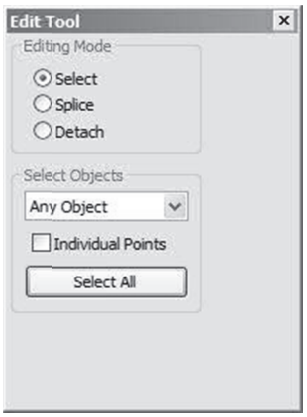
For more information on Markers, please see the chapter on Markers, starting on page 55. While in Editing mode, you can right-click on a marker in the tracing window to display a list of actions you can perform.

Selecting A Marker Or Markers

You need to select a marker or markers before you can perform an action.

To select a marker or markers

1. Click **Edit Select Objects** or click the **Select Objects** button. Stereo Investigator puts you in Edit Mode and displays the **Edit Tools** panel.



2. With **Select** selected under **Editing Mode**, select **Only Markers** from the **Select Objects** drop-down list box.
3. Click on a marker to select it. Stereo Investigator highlights the selected marker.
4. To select another marker, click on it. Stereo Investigator deselects the currently selected marker and selects the new marker.
5. To add markers to those already selected, hold down the SHIFT key and click on the markers to select.

6. Holding down the CTRL key allows you to add or remove markers from a group of selected objects. Clicking on an unselected marker adds it to the selected group, clicking on a selected marker removes it.
7. Click **Select All** to select all markers.

Editing Markers

After selecting a marker or markers, right-click and choose a command from the menu.

- **Change to Marker Type**: Displays a dialog where you can choose a new marker type.

Marker Type

It is important to understand the concept of Marker Type before editing marker properties. A marker type is defined as all markers having the same shape. The shape of a marker cannot be changed without changing its type. Click **Display>Display Preferences>Marker** tab to see all marker types available. These are the same marker types that appear in the marker toolbar. All markers of a given type are grouped together for changes made from this page.

If a marker name is changed in the editing mode, the marker remains the original type, but is assigned to a sub-category by name in the analysis categories. This is different from the functioning of the contour names, where contours with identical names are assigned automatically to the same type.

- **Move Selected Marker**: Turns the cursor into a hand. Click and drag the marker to a new location.
- **Rotate Selected Marker**: Rotates the marker or group of markers around the center point indicated by a green gunsight icon. If a single marker is selected, this option has no effect unless the center is moved. If several markers are selected, they rotate around a point in the center of the grouping. The icon marking the axis of rotation can be moved to a new location by dragging it. The markers cannot be rotated around their own centers, for instance to turn them sideways.
- **Flip Selected Marker**: Displays a dialog which you use to select a horizontal or vertical flip for the marker. Stereo Investigator flips the marker around the center defined as the reference point. If you uncheck **Flip around origin (reference point)**, Stereo Investigator flips two or more markers only, using their center as the origin.
- **Delete Marker**: Deletes all selected markers.
- **Hide Selected Marker**: Hides the selected markers. See Hidden Objects for information on working with hidden objects.
- **Copy Selected Marker**: Copies the selection to the **Clipboard**.
- **Place Markers Into Set**: Displays the **Name of Set** dialog box. Type a name for the set and click **OK**. Stereo Investigator places the markers into a set.
- **Change Marker Color**: Displays the Color dialog box, which you use to choose an existing color or define a new one.
- **Rename Selected Marker**: Lets you rename the selected markers.
- **Modify Z Position**: Prompts you to either **Shift Z Values** or **Set Z Values**. **Shift Z Values** allows you to move the marker up or down (by specifying a positive or negative number) a given

number of microns. **Set Z Values** allows you to tell the program the correct Z value for that marker. If multiple markers are selected, **Set Z Values** sets them all to the same Z value.

- **Modify Intrinsic Marker Size**: Using the **Modify Marker Size** dialog box, you can modify the intrinsic size of a given marker in three ways: 1) A slider bar allows for dynamic change to the intrinsic size measurement. The value you have selected is shown in **Set**, 2) Use **Set** to specify a new value, or 3) Use the **Scale** to specify a factor by which the intrinsic size should be changed (i.e.: specifying 3 increases the thickness by 3X its current value, a 5mm thick marker becomes 15mm thick). The new value does not dynamically change in **Set** if **Scale** is used, but is changed upon exiting the dialog box. For more information on setting marker size, see the discussion of marker size in Placing Markers.
- **Apply Shrinkage**: This is equivalent to using the **Tools>Shrinkage Correction** command, allowing you to apply shrinkage to a single marker, or to the entire section, if you used **Edit>Select All Objects** to enter the Editing Mode, or if all tracing objects have been selected. **Apply Shrinkage** is most commonly used when flipping sections mounted upside down (Flip a single section). **Apply shrinkage** can be used to correct for known amounts of shrinkage, if you want measurements to reflect the parameters of the tissue before processing. However, this would usually be applied to an entire data stack, rather than to just a few markers.

A Note on Shrinkage Factor: The shrinkage factor acts as a multiplier, and should be the inverse of the actual change in size. If you know that your tissue has shrunk to 25% of its original size, the factor to apply is 1/0.25, or 4. However, if you have a 25% shrinkage, the tissue is now 0.75 of its original size. Therefore, the factor you would apply is 1/0.75, or 1.33.

- **Place Marker in Section:** (Only available if using sections). Places the selected markers into a new section. When this option is selected, Stereo Investigator displays a dialog box which you use to selected the section to place the marker.
- **Mark as Locus**: Designates a marker as a locus or markers as loci. Used by Neurolucida Explorer when performing a locus analysis, measuring the straight-line distance from the locus/loci to all other markers in the file.. See the Neurolucida Explorer Help file for information.
- **Attach Markers to Object**: Lets you bind a marker or set of markers to an object. Later manipulations of the object include the attached markers. A good application of this tool would be to attach boutons that you have marked to their axon. The axon can then be moved, rotated or scaled while keeping the markers in their appropriate locations. To use this tool, first select all markers that you want to attach. After selecting **Attach Markers to Object**, Stereo Investigator prompts you to click on the tree or contour that you would like the markers attached to. Note that this tool attaches markers to a specific branch of a neuronal process, so click *directly* on the appropriate segment.
- **Undo Last Type Change**: Undoes the last change to a marker.
- **Exit Selection Tool**: Returns you to the previous mode.

Chapter 7

Markers

Marker Properties And Combination Markers

To work with markers, you click on a marker in the **Markers** toolbar to select or deselect a marker. To make changes to markers, right-click on the marker toolbar and choose a command from the menu.

Showing Marker Summary And Names

You can display both marker name and a summary of how many markers have been placed.

To display the marker summary

- Right-click on the **Markers** toolbar and choose Show Marker Summary.

To display marker names

- Right-click on the **Markers** toolbar and choose Show Marker Names.

Changing Marker Attributes

To change a marker color

1. Right click on a marker in the **Markers** toolbar and choose **Change Marker Color**. Stereo Investigator displays the **Color** dialog box.
2. Pick a new color, and click **OK**.

Stereo Investigator changes the color for that marker, including markers already placed.

To change a markers size

1. Right click on a marker in the **Markers** toolbar and choose **Resize Marker**. Stereo Investigator displays the **Resize Marker** dialog box.
2. You can change the size in pixels, in microns, or by an intrinsic value. Choose one, type a required measurement, and click **OK**.
3. Stereo Investigator changes the size for that marker, including markers already placed.

To hide a marker

You can hide a marker—make it unavailable for placement and hide those already placed.

- Right click on a marker in the **Markers** toolbar and choose **Hide Marker**.

Stereo Investigator makes the marker unavailable in the toolbar and hides markers already placed.

To rename a marker

1. Right-click on a marker and choose **Rename Marker**.
2. In the **Rename Marker** dialog box, type a new name and click **OK**.

To unhide a marker

- Right click on a hidden marker in the **Markers** toolbar and choose **Hide Marker**.

Stereo Investigator makes the marker unavailable in the toolbar and hides markers already placed.

Working With Combination Markers

Combination markers combine 2, 3, or 4 markers into one marker. Use a combination markers for labeling cells and structured that are marked with two or more staining methods.

To create a combination marker

1. Right click on the **Markers** toolbar and choose **Define Combination Marker**.
2. Choose one marker from the top grid to represent 2 or more other markers.
3. Choose up to 4 markers from the lower grid. These markers are replaced by the marker from the top grid when you place the combination marker.

   Click on a marker to see if it has been defined as a combination marker. If it has, Stereo Investigator highlights the markers in the lower grid markers combined by this marker.

4. To record the definition of a combined marker, click **Apply** if you want to define more combination markers. Click **Apply and Close** accepts the current definition and closes the dialog box.

To mark with combined markers

1. Place *individual* markers as appropriate in your tracing. Use the markers selected in the lower box of the **Define Combination Marker** dialog box to designate cells seen with different staining protocols.
2. Go back through your tracing after the individual markers have been placed, and at each site where you determine that the markers are close enough together to represent double labeling of the same structure, place a *combined* marker (described in the following steps).
3. On the Markers toolbar, right-click and choose **Enable Combination Markers** or click **Enable Combination Markers**.
4. To place a combined marker, adjust the circular cursor (with the mouse wheel or + and - keys on the numeric keypad) so that it is large enough to encompass all markers to be combined. Select the combination marker from the marker toolbar. Each time you left click over 2 or more markers to be combined, the markers are replaced with the combination marker.

If you left click and the circular cursor does not encompass all the individual markers that make up the definition of the combined marker, the combined marker is still placed but no individual markers are removed.

The individual markers that have been combined are removed from the marker totals and replaced with the new combined marker. It is up to you to keep track of what the combined markers represent, and remember that when a combined marker is listed in Marker Totals in Neurolucida Explorer, it actually represents two or more other markers.

This function is not available if you have interrupted tracing of a contour or tree to place markers. All trees and contours must be complete for the combination marker tool to be available.

Changing Markers Already Placed

To change markers to another type

1. Right click over the marker type to change on the **Markers** toolbar and choose **Change Traced Marker**.
2. In the **Change Marker Type**, click on the new marker type and click **OK**. All existing markers of the selected type are changed to the new type. If the color was changed previous to this operation, the new color is preserved.

This operation only effects markers that were already placed. Any new markers maintain their original type.

To delete markers

1. Right click over the marker type to delete on the **Markers** toolbar and choose **Delete All Markers of This Type**. Stereo Investigator deletes all instances of this marker.

Placing Markers

Placing markers acts is a separate mode of operation within Stereo Investigator. While a marker is selected, the ability to trace contours or neurons is blocked. In order to exit the Marker mode, click a second time on the Markers button.

Selecting Markers

Click to select a marker on the **Markers** toolbar along the left edge of the tracing window. To choose a different marker, click on it. Click **Undo** to delete markers in the reverse order of their placement. However, once you exit Marker mode by clicking an already depressed marker icon, the ability to delete markers with the Undo button is lost; at this point you must use the Editing Mode to delete them.

Mapping Markers Of Choice

Here are some hints for working with markers:

- If the area being mapped is larger than the field-of-view, turn on AutoMove (click **Move>AutoMove**).

- If you make a mistake, **Undo** erases the last drawn marker one by one back to the first one placed when the marker toolbar was activated.
- While placing markers, you can use **Joy Free** or **Joy Track** to move the stage. Remember that if you use **Joy Free**, you lose registration between the specimen and the tracing. Stereo Investigator returns you to Marker mode when the joystick mode is finished.

Attaching Markers To A Contour

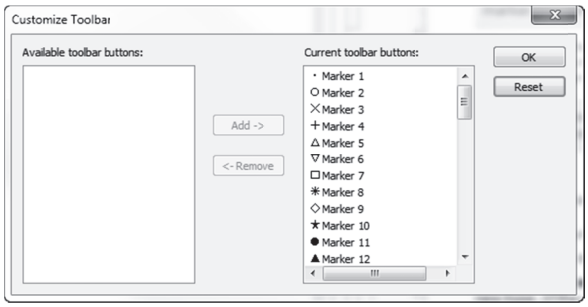
- If you select a marker while of a contour, but before the contour has been completed with either **End Open Contour** or **Close Contour**, Stereo Investigator attaches the markers to that contour. This provides analysis information in Neurolucida Explorer, and also allows for editing operations to be applied to the contour and marker simultaneously.
- if you want to attach markers to a contour after the contour has been completed, select all appropriate markers in the Editing Mode, right click, and choose **Attach Markers to Object**. .

Customizing the Markers Toolbar

You can add or remove markers from the Markers toolbar.

To add or remove markers from the toolbar

1. Right-click on the Markers toolbar and choose **Customize Markers** bar. Stereo Investigator displays the **Customize Toolbar** dialog box.



The current buttons are in the right column. Any buttons you can add are in the left column.

2. Select the button or buttons to add or remove and click the appropriate button.
3. Click **OK** when done.

Chapter

8

Automating Your Acquires

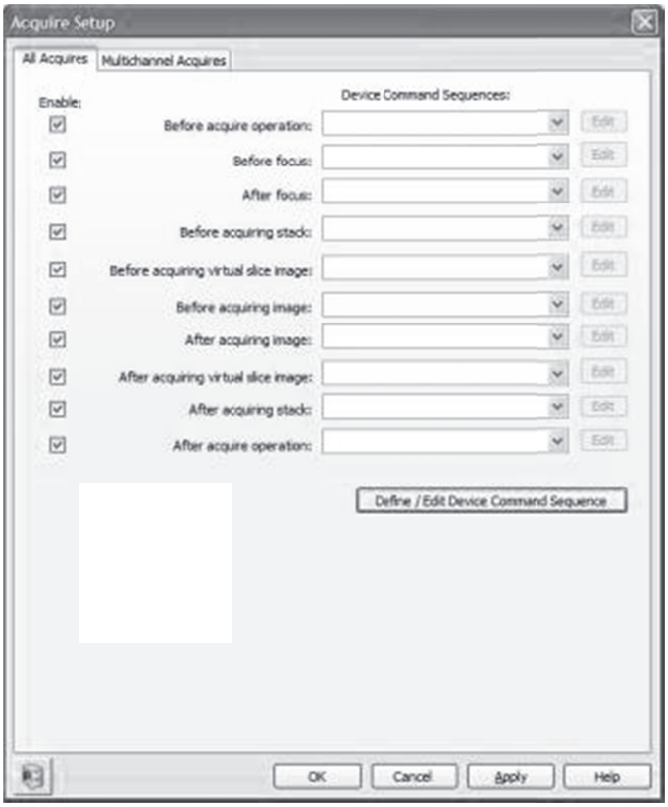
You can build complex commands for your acquisitions with the Acquire Setup command, automating repetitive tasks associated with the **Acquire Image, Acquire Multi-channel Image, Acquire Virtual Slice,** and **Acquire Image Stack** operations.

Automating Your Work

You can set commands and options for all acquires or for multi-channel acquires using Device Command Sequences and Messages. These are commands performed by devices connected to your system. You can have the computer alert you before or after an action, change lenses, move the stage, etc. For more information see Message Device Setup and Device Command Sequences Setup.

Set options for all acquires

Choose this tab to set options used by all acquires.



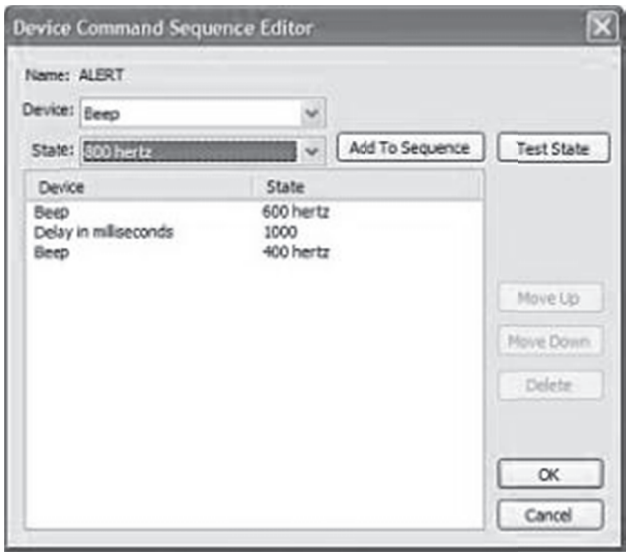
This tab has the following options:

- **Enable:** Check to enable this command sequence; uncheck to disable this command sequence.
- **Device Command Sequences:** The Device Command Sequences start with commands you with to perform before an acquire begins and ends with commands to perform after the acquire operation completes. The sequences in order are:
  - Before acquire operation
  - Before focus
  - Before acquiring stack
  - Before acquiring virtual slice image
  - Before acquiring image
  - After acquiring image
  - After acquiring virtual slice image
  - After acquiring stack
  - After acquire operation
- **Edit:** Click to edit an existing command sequence available from the drop-down list.
- **Define/Edit Device Command Sequence:** Use to create a new command sequence or edit an existing sequence.

To create a new command sequence:

1. Click **Enable** next to the command sequence you wish to activate.
2. Click **Define/Edit Device Command Sequence**. Stereo Investigator displays the **Device Command Sequences** dialog box.

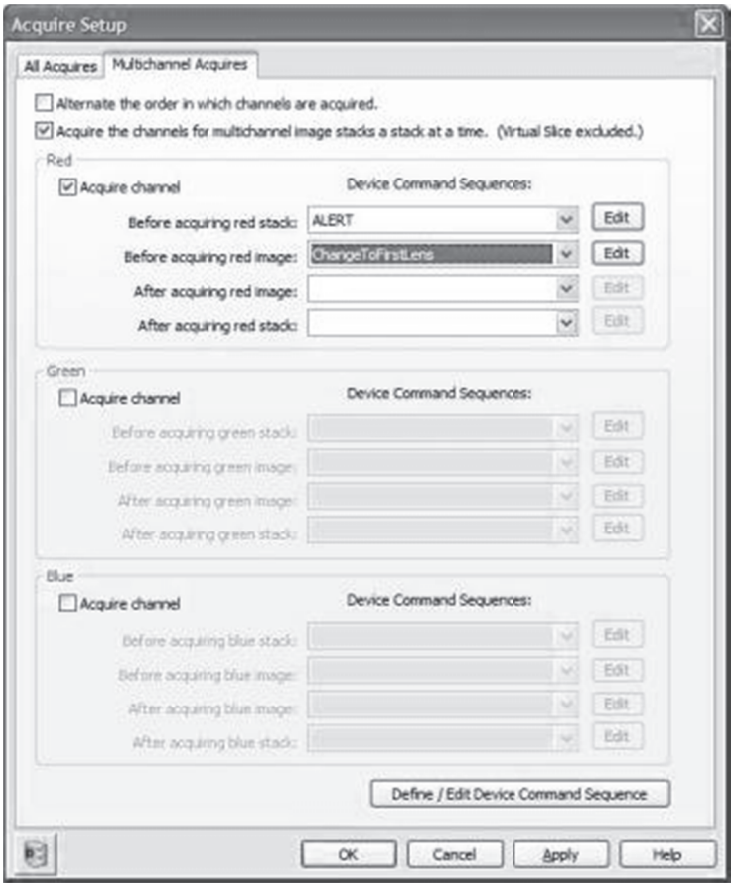
3. Under **Add Device Command Sequence,** type a name for this command sequence and then click **Add**. Stereo Investigator displays the **Device Command Sequence Edi**tor dialog box.



4. Select a **Device,** then select a **State** for the device. For example, you can select *Beep* as the device and *300 hertz* as the state.
5. Click **Test Sequence** to try out the command
6. Click **Add to Sequence.** Stereo Investigator adds the command to the stack.
7. When you are through adding or editing commands, click **OK** to return to the **Acquire Setup All Acquires** dialog box.
8. Continue adding command sequences as needed. When finished press **OK.** Stereo Investigator saves your changes.

Set options for multichannel acquires

If you are performing multi-channel acquires, you can set different command sequences for each channel using the **Multichannel Acquires** tab.



This tab has the following options:

- **Alternate the order in which channels are acquired:** Direct Stereo Investigator to alternate the order in which channels are acquired.
- **Acquire the channels for multichannel image stacks a stack at a time (Virtual Slice excluded):** Acquire each channel for multi-channel image stacks one stack at a time (i.e., acquire the Red stack, then the Green Stack, then the Blue Stack).
- **Acquire channel: S**elect (check) the Acquire channel check box for each color channel you want to acquire. For example, if you are using dyes that fluoresce on the Red channel and Blue channel, you can tell the system to skip acquiring the Green channel.

If this box is clear (unchecked), that channel will not be acquired. You don't need a command sequence for each channel to acquire it, but you must select (check) the box to acquire the channel.

As with an **All Acquires**, the devices and commands available depend on the specific hardware you have installed.

- **Device Command Sequences:** The Device Command Sequences in order are:
    - If you are going to acquire the channel by image (red, green, blue, then move onto next location), the command order is for each color channel as follows:
        - Before acquiring image

    - After acquiring image
- If you are going to acquire the channel by stack (red, green, blue) the command order is:
    - Before acquiring stack
    - Before acquiring image
    - After acquiring image
    - After acquiring stack

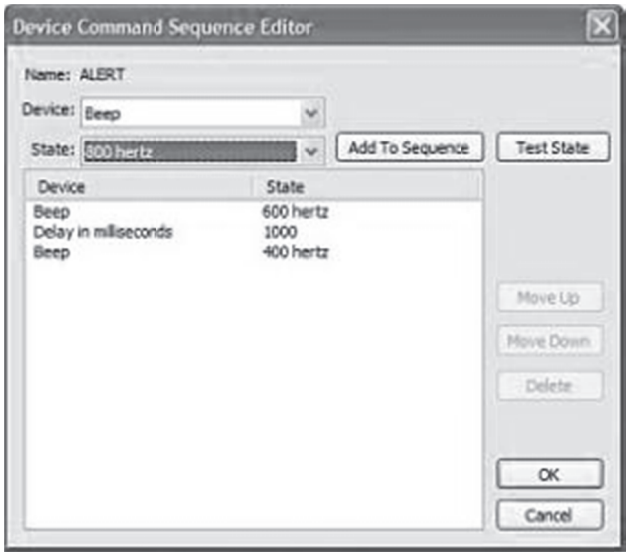
Edit: Click to edit an existing command sequence available from the drop-down list.

Define/Edit Device Command Sequence: Use to create a new command sequence or edit an existing sequence.

To create a new command sequence:

1. Click **Enable** next to the command sequence you wish to activate.
2. Click **Define/Edit Device Command Sequence**. Stereo Investigator displays the **Device Command Sequences** dialog box.

3. Under **Add Device Command Sequence,** type a name for this command sequence and then click **Add**. Stereo Investigator displays the **Device Command Sequence Edi**tor dialog box.



4. Select a **Device,** then select a **State** for the device. For example, you can select *Beep* as the device and *300 hertz* as the state.
5. Click **Test Sequence** to try out the command
6. Click **Add to Sequence.** Stereo Investigator adds the command to the stack.
7. When you are through adding or editing commands, click **OK** to return to the **Acquire Setup All Acquires** dialog box.
8. Continue adding command sequences as needed. When finished press **OK.** Stereo Investigator saves your changes.

Chapter 9

The Image Stack Module

The Image Stack module is an extension to the standard version of MBF Bioscience software, providing an additional capability of acquiring data from confocal image stacks. The confocal module accepts confocal image stack files in the Biorad composite .pic format, the Olympus Fluoview format, the Zeiss LSM format, as well as a stack of images in a series of bitmap files, i.e., tiff, jpeg, etc. All of the capabilities of the standard Stereo Investigator program are also available.

The Image Stack module allows you to focus through an image stack in real-time. You may specify the focal distance between image planes. The software automatically keeps track of the depth (Z-axis) values while you trace. This allows you to perform tasks such as 3D neuron reconstruction with the same ease as with a standard brightfield microscope. All data collected with Stereo Investigator Image Stack module can be analyzed, displayed, and rotated with the Stereo Investigator Explorer program. There is no limit to the number of images in a stack, except as constrained by your computer memory.

What file formats are supported?

We support the following formats:

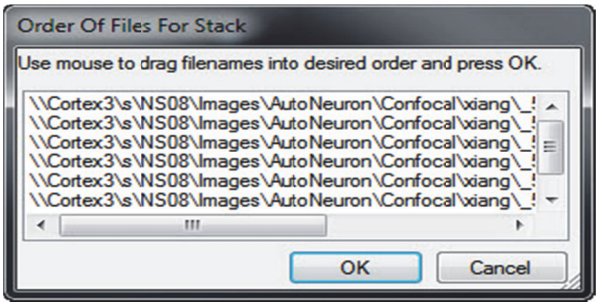
MBF JPEG2000 (.jp2; .jpx; .jpf)	MBF Tiff (.tif; .tiff)	JPEG2000 (.jp2; .jpx; .jpf)
Tiff (.tif; .tiff)	Bit Map (.bmp)	JPEG (.jpg, .jpeg)
ZSoft (.pcx)	PNG files (.png)	TARGA files (.tga)
Olympus Fluoview (.tif)	Portable Image (.pgm; .pbm; .ppm)	BioRad Confocal Image (.pic)
FlashPix (.fpx)	Zeiss Confocal LSM (.lsm)	Zoomify (.pff)
DICOM (.dcm)	ANALYZE (.img)	NanoZoomer (.ndpi; .vms; .vmu)
Aperio SVS (.svs)		

How do I load image stacks?

You open Image Stacks in much the same way you open other file.

To open an image stack composed of several files

1. Click **File>Image Stack Open**. Stereo Investigator displays the **Image Stack Open** dialog box.
2. Select the images and click **Open**. Stereo Investigator displays the **Order of Files for Stack** dialog box.



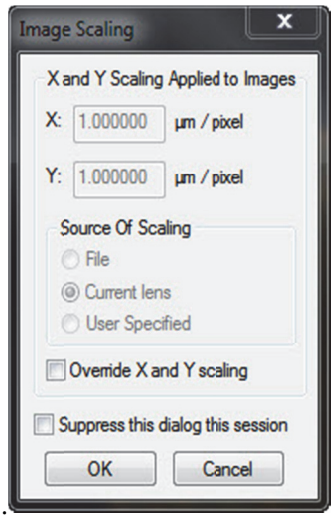
3. If the files are not in the proper order, you can drag them in the list until the order is correct.
4. Click **OK**. Stereo Investigator displays the **Image Scaling** dialog box.
   Since single image files don't contain Z spacing information, you need to manually enter this information. The program prompts you to enter the image separation while loading the stack. This is the distance between images. You can use the focal distance or the physical distance.
   - **Focal Distance**—Image stacks collected with Stereo Investigator are collected using spacings that describe the focal plane separation.
   - **Physical Distance**—describes the physical movement of the microscope stage as images are collected. If you select this option, correction factors must be applied to convert the microscope movement into the movement of the focal plane. The X and Y dimensions of the imported image default to the micron/pixel ration for the current lens. Select the lens that was used to capture the images before loading the image stack. If the image stack was collected on a different microscope it is important to calibrate a lens for that system. Select that lens before loading the image stack. For more information, please refer to the section Calibration for Imported Images.
     If you select this option, you need to select the correction factor for the physical distance between the lens and the image. Stereo Investigator automatically enter this value for **Air, Oil,** and **Water**. If you select **Other,** you must manual enter the factor.

4. You can use the X and Y scaling used when the image was acquired, or override it. Click Override Z and Y scaling, choose the source, and then enter the values.
5. Click **OK**. Stereo Investigator loads the image stack.

If you load an image for which there is no matching lens, Stereo Investigator prompts you to define a new lens to match the image scaling. For information on defining a lens, see Defining lenses.

To load an image stack file that contains all the images

1. Click **File>Image Stack Open**. Stereo Investigator displays the **Image Stack Open** dialog box.
2. Select an image file, and click **Open**.
3. Click **OK**. Stereo Investigator displays the **Image Scaling** dialog box



Stereo Investigator needs this information if it isn't contained in the image file.
The program prompts you to enter the image separation while loading the stack. This is the distance between images. You can use the focal distance or the physical distance.

- **Focal Distance**—Image stacks collected with Stereo Investigator are collected using spacings that describe the focal plane separation.
- **Physical Distance**—describes the physical movement of the microscope stage as images are collected. If you select this option, correction factors must be applied to convert the microscope movement into the movement of the focal plane. The X and Y dimensions of the imported image default to the micron/pixel ration for the current lens. Select the lens that was used to capture the images before loading the image stack. If the image stack was collected on a different microscope it is important to calibrate a lens for that system. Select that lens before loading the image stack. For more information, please refer to the section Calibration for Imported Images.
  If you select this option, you need to select the correction factor for the physical distance between the lens and the image. Stereo Investigator automatically enter this value for **Air, Oil,** and **Water**. If you select **Other,** you must manual enter the factor.

4. You can use the X and Y scaling used when the image was acquired, or override it. Click **Override Z and Y scaling**, choose the source, and then enter the values.

5. Click **OK**. Stereo Investigator loads the image stack.

If you load an image for which there is no matching lens, Stereo Investigator prompts you to define a new lens to match the image scaling. For information on defining a lens, see Defining lenses on page 18.

Image stacks and lenses

The X and Y dimensions of the imported image default to the current lens. Select the lens that was used to capture the images before loading the image stack. If the image stack was collected on a different microscope it is important to calibrate a lens for the remote system. Select that lens before loading the image stack. See Calibration for Imported Images for more information.

Image order and nomenclature

A multiple image file such as a PIC or TIF file contains all of the images for one image stack. The images are interpreted to be sequential images with the first images the topmost images and subsequent images are placed at decreasing Z positions. The first image is placed at the current Z position.

The order in which multiple images from single-image files are loaded is determined by the order of the files in the dialog box. Therefore, we recommend using a naming convention that orders the images appropriately. For example, if you number a set of images *image1*, *image2*, *image3*, etc., image10 is placed after image1 but before image2. Instead, you should number the images as follows: *image01*, *image02...image10*, *image 11...* in order to maintain the correct order. The images are loaded in the same order as the files appear in the **File>Open** dialog box.

See the section Serial Sections from Imported Image Files on page 77 for more information.

Viewing image stacks

The top image of a stack is loaded at the current Z position of Stereo Investigator. When the stack is loaded, the top image of the stack is displayed. A message in the status bar reads "x of n images" where x is the number of the currently displayed image and n is the total number of images in the stack. To navigate through the stack, use the Page Up and Page Down keys on the keyboard

Multiple adjacent image stacks

Using the Spatially Organized Framework for Imaging (**SOFI**) technology, multiple image stacks may be positioned in 3D space. This allows data acquisition of specimens larger than a single field-of-view.

To load and position multiple image stacks, the Image Organizer and Move Image function are used:

Load the first stack of the series. At this point, you can move (using Move arrows or **Move>Move To**) to the approximate position of the new stack, or load the new stack in the same position as the first stack and move it later. Load the second stack of the series. The second stack will load with the first image of the stack at the same Z level as the currently open stack. To load images at the same Z, be sure the first stack is showing the first image of the stack. The status bar will read "Image 1 of n". To offset images, move the open stack to the level at which you would like the second stack to begin. For example, if the second stack was acquired starting 3 microns below first one, and the image separation is one micron, move the open stack to "Image 3 of n" before opening the second stack.

To position the second stack, open the Image Organizer, and be sure that only the second stack is checked (a red checkmark shows in the second column). Next, select the Move Image tool, and adjust the position of the second stack relative to the first.

To change the position of one of the stacks in the Z direction, select the stack in the image organizer (so that the selected stack is highlighted in black in the last column). Close the Image Organizer. With Move Image selected, use the Page Up and Page Down keys to navigate through the stack until the stack matches up with adjacent stacks.

Deselect **Move Image**, and load more stacks as described above, or begin tracing.

At this point, tracing can be done as if you were tracing from a live image. Focus through the stacks using the Page Up and Page Down keys, and navigate in the X- and Y-axes using Move>Move To, Move>Go To, the field movement buttons, or Move Image and Tracing.

When the data file is saved, the relative information about the position of the image stacks are also saved, so the next time the data file is read in, the images are put in their proper position.

Opening and merging multiple adjacent image stacks

To open a single multi-channel image stack with each channel displayed in a different color, select **File>Image Stack Merge and Open**, and select the multi-channel file from the Open Image Stack dialog box. You are presented with a Select Desired Color Channels dialog box.

If a multi-channel image stack is selected, the same file name appears in each of the Confocal Stack fields (as shown above). Use the Image Channel fields to specify which channel appears in Red, Green, or Blue (as indicated by the color name at the left of the dialog box). Any color channel can be left blank by selecting none from the Confocal Stack field.

Merging Multiple Single Channel Image Stacks

To open and merge multiple single-channel image stacks, use the SHIFT key and the left mouse button to select all desired image files from the Open Image Stack dialog box. The Select Desired Color Channels dialog box appears. In this case, designate the color for each separate file by selecting the different image file names from the Confocal Stack fields. The Image Channel fields should remain blank, as each file contains only one channel. Any color channel can be left blank by selecting none from the Confocal Stack field.

Saving image stacks

If you have modified an image associated with a stack of images, use this menu option to save the images as a new file. This window also automatically opens following the **Imaging>Acquire Image Stack** operation.

Tracing from image stacks

When tracing from confocal image stacks, trace as if you are working with a single tissue section. Stereo Investigator keeps track of the Z-depth of your tracing by recording the Page Up and Page Down movements through the sections. It is not necessary to use the Serial Section Manager, as the program treats the different images of the stack like different focal depths of the same section.

When working with multiple adjacent image stacks, some users prefer to trace all visible structures in a given stack before moving to the next adjacent stack; others prefer to follow a structure through the stacks while tracing. If you choose to trace all parts of a neuron or other branching structure in one stack before moving to the next, you need to add to the endings of the incomplete processes.

Chapter 10

The Serial Section Manager

Stereo Investigator has been designed to facilitate serial section reconstruction of light and video microscopy sections, EM photomicrographs, image stacks, and trans-illuminated negatives. The depth separation between sections can range from a fraction of a micron to hundreds of microns. Sections can be contiguous or non-contiguous, and can be oriented at different angles on specimen slides or data tablets.

The serial section reconstruction procedure allows you to specify the depth separation between consecutive sections, and allows you to properly align the sections.

The Serial Section Manager allows data files traced in Stereo Investigator to be reconstructed for full volumetric analysis and solid representation using Neurolucida Explorer. Using Stereo Investigator for stereological analysis of tissue does not require any special alignment of tracings for accurate data collection. However, in generating 3D solid objects, care must be taken to align sections to create a smooth surface on the solid representation. Since the procedure for tracing from slides differs slightly from that for tracing photomicrographs, camera lucida drawings, MRIs, etc., users should refer to the instruction sections Serial Sections from Imported Image Files or Serial Sections from a Data Tablet if you are not tracing from slides.

We designed this feature to facilitate serial section reconstruction of light and video microscopy sections, EM photomicrographs, confocal stacks, and trans illuminated negatives. The depth separation between sections can range from a fraction of a micron to hundreds of microns. Sections can be contiguous or non-contiguous, and can be oriented at different angles on specimen slides or data tablets.

The serial section reconstruction procedure allows you to specify the depth separation between consecutive sections, and allows you to properly align the sections.

The Coordinate System

Stereo Investigator employs a right-handed Cartesian coordinate system. When you focus down through a section the depth value increases in the negative direction. Thus, if sections are 10mm thick, and the top of the section is at a depth of 0, the bottom of the section is at a depth of -10mm.

Fiducial Points

Fiducial points are points on your specimen that you mark to help you align subsequent sections. Fiducial points, then, should be points that are present in all sections of the structure you are reconstructing. If the tissue has a cut edge, a point along this line can be a fiducial point. Other examples of good fiducial points include points along the central canal of the spinal cord, the central sulcus of the brain (in coronal

sections), or the aorta in transverse embryo sections. Generally, any structure that runs longitudinally through the entire structure you have sectioned lends itself to the placement of good fiducial points.

If there is not a structure that is present in all sections, as is often the case, it is recommended to mark fiducial points on each section that are also found on the next section. Then each section is matched with the tracing of the preceding tracing, not the first tracing.

The best placement of fiducial points is done by selecting the points at a low magnification, then going to a higher magnification to adjust the placement of the points as accurately as possible.

Note that the alignment of sections is a bit of a subjective process. Fiducial points are just another tool to help you do it as accurately as possible. If their placement is not precise, they are still of help.

Preparing Tissues for Serial Section Reconstruction

3D reconstruction through multiple sections is far easier if your tissue is sectioned and prepared in a systematic fashion. Below are guidelines to make progress simple and fast. Note that if you have not followed these guidelines, you can still do serial section reconstructions. One of the strengths of Stereo Investigator is that you can flip sections mounted upside down, and use shrinkage corrections to compensate for many irregularities.

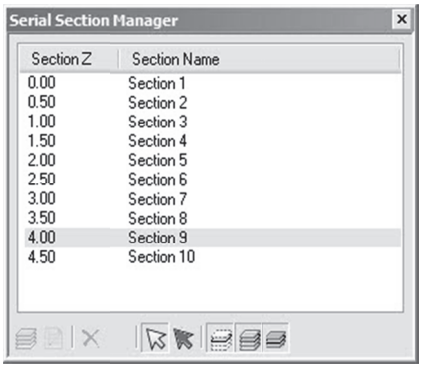
Guidelines

- Have all sections aligned in the same orientation. This can be done by sectioning directly onto slides or by making some kind of identifying mark in symmetrical tissues that are going to be processed free floating. Try to have all sections mounted in the same Z orientation (right side up). It is much easier to use Stereo Investigator to flip the occasional upside-down section than to depend on that tool to orient all of your sections.
- Have sections mounted in order on slides. The bottom of one section is aligned with the top of the next section to insure continuity of tracing.
- Mount every section when applicable. While this is not necessary for 3D reconstruction of large solids, you cannot reconstruct neurons without every section.
- Keep track of any missing sections in your notebook. It can be very frustrating searching for the ‘next’ section during neuron reconstruction if it is not there.
- Don’t try to mount too many sections per slide and don’t try to mount them too close to the edges of the slide (especially the left and right edges): you may not be able to trace all the way to the edge of the slide without the condenser cap hitting the stage.
- Pick a good naming convention for your slides and sections. If you are not mounting every section, include the logical and actual section number in the section name. For instance, if you are mounting every 5th section cut, the postfix for the first mounted section could be 1-1, while for the second mounted section it could be 2-6, indicating that it is the second actual mounted section, but the 6th section of the exhaustive (logical) series. It can also be useful to encode such information as the experiment, group, animal, and slide # in each section name, although this information can also be stored in the file description for the entire file.

The Serial Section Manager Dialog Box

You use the Serial Section Manager to define the key parameters for each section (such as the initial section thickness, the current average mounted thickness, Z position, etc.) before you sample from them.

When performing stereological probes through volumes of interest that extend over many sections (such as the Optical Fractionator for estimating cell populations), the Serial Section Manager stores information about all of the sampled sections. In order to perform the calculations for computing the desired estimate and its CE (Coefficient of Error), information about the sampling performed and information about the sections is necessary. The Serial Section Manager records information about the sections and makes it accessible during calculation of your estimate.



The Serial Section Manager

- **Section Z:** This column shows the depth value associated with each section. This is usually the Z coordinate for the top of the section. Data contained within a section is not restricted to the Z range of that particular section. Data points placed in a section can have any Z value, but generally the data will make more sense if the Z values are restricted by the user to those contained within the section.
- **Section Name:** This column shows the name assigned to each section; either the default name, or the name that has been assigned by the user.

Buttons

- **New Section:** Adds a new section.
- **Edit Section:** Lets you edit the properties of the selected section.
- **Delete Section:** This option deletes the currently selected section. The locations of the other sections are not changed with the deletion of a section.
- **Delete Other Sections & Data:** This option supports the use of atlasing templates. The Serial Section Manager can be used to page through a series of atlas templates and select the one most appropriate for the current tissue section. Once the best fit section is identified, all other sections can be deleted using the Delete Other Sections & Data button. A second dialog box requesting confirmation appears before the sections are deleted. There is not an Undo function for this operation.
- **Select by Section:** Selects contours by section. Click a section in the list and then click **Select by Section**.

- **Select Objects Not in a Section:** You must first click **Select by Section** to use this command. Sometimes you will have objects that aren't in any section. Use this command to select them for editing.
- **Display Current Section Only:** Displays just the current section in the list.
- **Show Suppressed as Gray:** Displays suppressed objects as gray.
- **Display Flanking Sections:** Displays the sections immediately above and below the current section. For example, if section 9 is active, this command shows section 8 and section 10 as well.

Setting Up The Serial Section Manager And Tracing

Before sing the Serial Section Manager, you need to set its options. The instructions here will help you .

Setting Up *Before* Tracing

These instructions are for the set-up of a serial section reconstruction using the microscope and specimen slides.

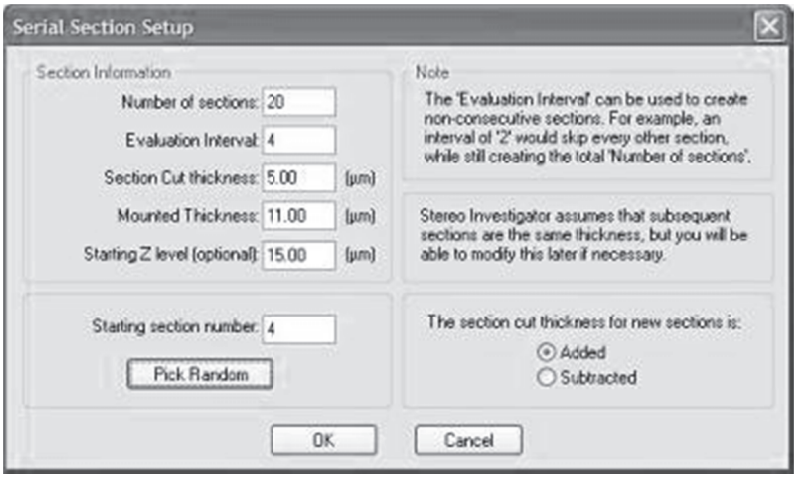
Do not start tracing the first section until you have gone through the following set-up procedures.

If you are reconstructing serial sections from imported images (digital images of specimens, MRI images, or confocal stacks) see the appropriate section for specific set-up instructions.

1. Click **Acquisition>Live Image** to display a live image. Be sure that the lens selected in the Lens Selection Menu is the same as the lens selected on the microscope turret. Move the stage to select an appropriate reference point. The reference point is only applicable to your first section, as it can be moved during subsequent alignment functions. However, if you select a structure that is present in most of your sections for your initial reference point, it can be helpful in placing the specimen in the correct location when replacing a slide on the microscope.

   After selecting a reference point, you may want to switch back to a relatively low power lens so that as much of the region of interest as possible is in your field-of-view.

2. Focus on the top of the current section.
3. Click **Tools>Serial Section Manager**. Stereo Investigator displays the **Serial Sections** dialog box. If this is the first time you are using the Serial Section Manager, no sections are displayed. You must set up the **Serial Section Manager**.
4. Click **New Section**. Stereo Investigator displays the **Serial Section Setup** dialog box.



5. Fill out the dialog box with the desired options.
6. When you are satisfied with the options, click **OK.** Stereo Investigator displays the Serial Sections dialog box with your newly defined section highlighted. Any new tracings made at this time are added to that section, and are added at the appropriate Z-level for that section.
7. Click **Close** to return to the tracing window, with the newly defined section as the active section.

Setting Up *After* You've Started Tracing

It is easy to forget to set up the Serial Section Manager until after the first section has already been traced. You find yourself ready to move to the next section and realize that the first section was never defined. Luckily, this is easily remedied by following steps below:

1. Perform the Serial Section Setup as described in steps 5-7 in "Setting Up the Serial Section Manager Before Tracing" on page 74. Your tracing will not be visible if Show Current Section Only is selected. Go to **Options>Display Preferences>View** tab and deselect **Show Current Section Only**.
2. Click **Edit>Select All Objects** to select all elements of your tracing.
3. Right click in the tracing window, and select **Place Objects in Section**. There is only one section to choose from; click on this section in the **Select Section** dialog box
4. All selected objects are placed in the newly defined section. Further tracing in this section can now be done.
5. If desired, turn **Show Current Section Only** back on.

This procedure is also used to add tracings to any section. Be sure that the section you are adding to is currently the Active Section in the Serial Section Manager.

Tracing Serial Sections

After setting up the Serial Section Manager, you begin the tracing of your specimen. The tracing itself is identical to that described in the sections on Tracing Contours. Note, however, that the serial section manager is not necessary for tracing neurons in several sections. More detailed instructions are found in Tracing Trees in Serial Sections. Below are instructions for moving from section to section and insuring proper alignment between sections.

Note on Terms: In the following discussion, Section A refers to the section currently being traced or which has just been traced; Section B is the next section to be mapped. Our discussion assumes that sections are mapped in ascending serial order. It is not necessary to proceed in this way but it is usually easier and more convenient.

To Trace Serial Sections:

1. Select a contour type from the Contour toolbar, and trace Section A as completely as desired, adjusting the focus as necessary. Use a different contour type for each different anatomical region you plan to trace. If there are separate left and right components to an anatomical structure, such as ventricles, it is often convenient to define separate contour types for each, for instance Left Ventricle and Right Ventricle. It is often useful to trace the section outline, even if it is not needed in your final study. If this is done the **Macro View** window can be used to see where you are at all times and to move anywhere in the section quickly and easily using **Go To**.

Unless you are using a data tablet, you may change lenses at any time. Remember to always change the lens in the Lens Selection List when you change the physical lens.

2. It is a good idea to save your tracing frequently. Click **File>Save As** and save your file as either a .dat or .asc file.
3. Use **Joy Free** to move the slide to the next section. It is usually useful to switch back to a low power objective for the move and align steps.
4. Align Section B with the tracing of Section A as precisely as possible. You can find instructions in the section that follows, "Aligning Serial Sections" **below**.
5. Once the sections are well aligned, focus on the top of Section B, and click **Tools>Serial Section Manager** and click the **New** button. At this point it is usually easier to hide the previous tracings, i.e., make sure **Display Current Section Only** is pressed.

Remember, you do not need to open a new file for new sections. While it is possible to create a 3D reconstruction from information saved in different files, Stereo Investigator is designed to store all information for a reconstruction in a single file, and this is the easiest way for you to complete a reconstruction.

6. Trace Section B, then repeat steps 1-5 until you reach the last section of your specimen.

Aligning Serial Sections

The following instructions outline the procedure for aligning a new tissue section with the tracing described in Tracing Serial Sections on page 75. This procedure is used in tracing serial sections. Section A refers to the section that has just been traced, while Section B refers to the new section about to be traced.

1. Click **Move>Joy Free**. Move the stage with the joystick until Section B is aligned relatively well with the tracing of Section A. Focus at the top of the section and exit **Joy Free**.
2. You now need to further align the tracing of Section A with the specimen that is Section B. There are a few ways to do this, depending on the degree and type of misalignment, as described here:
    - **Tools>Match** provides a best fit between the tracing and new specimen based on the location of 2 to 99 pairs of corresponding points. This is the easiest method to obtain a quick, good fit between the image and the tracing. **Match** rotates and moves the

overlay, without skewing or altering it, to get the best match with your image. To carry out a match, specify the number of pairs you are going to use for matching the tracing with the section. For each pair, first pick a point on the overlay, and then pick the corresponding point on the image. Repeat this for subsequent pairs. If this requires moving the stage, use **Move>Joy Track**, the **Go To** function of the **Macro View** window, the arrow buttons on the toolbar, or **Go To** to move to the next pair of points. **Match** moves all sections whether displayed as visible or hidden.

- **Move>Align Tracing** moves the tracing in the X, Y and/or Z-axis, but does not rotate the tracing. When you select this option, instructions appear in the status bar prompting you to first pick a point on the overlay (tracing), then to choose the point on the specimen (Section B) where you want this point to appear. Refocus if necessary before clicking on the second point. **Align Tracing** moves all sections whether displayed as visible or hidden.
- **Tools>Rotate Tracing** allows for simple rotation around the reference point. This tool only rotates visible sections, so be sure that all sections are visible (**Options>Display Preferences>View** tab, deselect **Show Current Section Only**) if you are going to use this to align a new section. **Rotate Tracing** has the advantage that you can see the tracing move while you are making the adjustment. **Rotate Tracing** moves only sections displayed as visible.

Be sure that Display Current Section Only is not selected if you are using the Rotate Tracing option (that is, be sure all sections are visible). If only one section is visible, that section is rotated out of alignment with the previously traced stack of sections.

A note on the reference point: You may notice that after aligning the second section that the reference point is not in its original location with regard to the tissue. This is normal, and is due to the fact that align and match functions move the entire tracing not including the reference point. The reference point is only an accurate locator of a point on your tissue in the first section of a stack. When returning to subsequent sections after closing the file, you need to manually align the tissue with the stack of tracings.

Serial Sections And Imported Images

Imported image files can be in several formats, including a series of images acquired from several slide section. For confocal images, you don't need to use the **Serial Section Manager**—we treat the stack as a single section. Moving between images is treated as changing focal planes.

For tracing from a series of imported image files, follow the same steps as tracing from slide material.

When working from acquired images, you need to know before beginning: the distance between sections, OR the thickness of sections and periodicity of the sections from which the images were acquired. In addition, you need to calibrate a lens to the scale of the acquired images if they were not acquired from the system you are currently using. See Calibration for Imported Images for more information about calibrating a lens for imported images.

1. Place a reference point.
2. Open the **Serial Section Manager** and define the first section.
3. Open the first image.

4. Trace the first section as you would from a live slide image.
5. Save the file.
6. Open the image of the next section. The location of the new section is not important, as long as the previous tracing is aligned with the image of the new section according to instructions found in Aligning Serial Sections. The only difference in the procedure is that **Move Image** is used instead of **Joy Free**.
7. Align the previous tracing with the image of the next section.
8. Define a new section.
9. Trace the next section.

The serial section reconstructions have slightly less detail, as focusing through the tissue sections is not possible, and tracings tend to have a "stair step" appearance as you move from section to section. However, the steps to follow for moving from section to section are identical to those for working with slide material.

Using A Data Tablet With Serial Sections

You can trace from a photomicrograph by using a data tablet. If you have images that have been scanned or digitally photographed, there is no need to use a data tablet, as these images can now be imported directly into Stereo Investigator for tracing. See Serial Sections and Imported Images on page 77 for instructions on working with digital images.

How To Trace Serial Sections With A Data Tablet

1. Make sure you have calibrated a tablet lens based on the scale of the photomicrograph you are tracing, and select that lens in the lens selection list. See Calibrating a Lens for Objects Placed on a Data Tablet.
2. Set up the **Serial Section Manager** according to the instructions in Serial Section Set Up. Enter the distance between photomicrographs into the **Enter the Section Thickness** field of the **Serial Section Setup** dialog box.
3. Place the photomicrograph under the plastic cover sheet of the tab-let so that it cannot move while being traced.
4. Move the cross hair of the data tablet mouse (four button puck) until the cross hair lies on the desired reference point and click the yellow mouse button. The reference point is used primarily for locating your starting point in the first section. It should be a point that you can easily find again on the first image.
5. Follow the general directions for Tracing from Serial Sections.
6. Continue tracing, treating each new photomicrograph as a new section until you have reached the end of the tissue of interest.

Working With Serial Sections—Upside Down Mountings

Most of the time your work with Serial Sections will be straightforward. Occasionally, you may run into a section mounted upside down. You can correct for an upside down section before starting to trace the

section or you can correct an upside down section that has already been traced. Here's how to deal with these sections.

Tracing Upside-down Mounted Sections

If you discover the upside down section before you start tracing it, use this procedure. You are most likely to discover the upside down section before tracing has begun if your tissue is asymmetrical. In this case, you are unable to align the tracings of previous sections with the current section, and need to flip the tracings of the previous sections in order to align them with the new tissue section.

Start the following procedure before designating a new section in the Serial Section Manager.

An analogy to a loaf of bread can be used to describe this procedure: Imagine that you are putting a loaf of bread back together one slice at a time. If you come across a slice that is upside down, one way to put it on in the correct orientation is to take the entire loaf and bring it around to the other side of that slice. Then you can put the slice on the loaf in the correct orientation without flipping the slice itself. This is how we deal with an upside down section, by flipping all the other sections to the "other side" of the inverted section by inverting the Z values. Then, once the upside down slice has been added, the whole loaf, including the new slice, is flipped back around to continue adding slices (sections).

1. Deselect **Options>Display Preferences >View tab>Show Current Section Only** so that all sections are visible.
2. Click **Tools>Shrinkage Correction** and change the value of the **Z** field to *-1.0*. Also change the value of either the **X** or **Y** field to *-1.0*, depending on whether you would like to flip the section vertically or horizontally. Entering *-1.0* in the **X** field flips the tracing left to right around the reference point. Entering a *-1.0* in the **Y** field flips the tracing top to bottom around the reference point. If the reference point was not placed in the center of the section, the tracing may be flipped so that it is no longer in the field-of-view. Use **Move>Go To** or the **Go To** command in the **Macro View** window to move the tracing back into the field-of-view, and then use **Joy Free** or **Align** to carefully re-align the section.
3. Click **Options>Display Preferences >View tab>Show Current Section Only** to display only the last section traced. You can now align this section with the upside down tissue section. Align the tissue section with the tracing using techniques described in Aligning Serial Sections.
4. Define the new section using **Tools>Serial Section Manager**. Type in the nominal depth value for the bottom of this section in the **Top of Section Depth** field. To have the Z information within the section recorded correctly, it is also necessary to invert the sign of the Z step size. Go to **Options>Stage Setup>XYZ Stage Setup** and change the sign of the Z step size. Trace the upside down section normally.
5. When the section has been completely traced, flip all sections including the latest one right side up again. Make all sections visible by deselecting **Show Current Section Only**, then click **Tools>Shrinkage Correction** and change **Z** to *-1.0*, and either **X** or **Y** (the same one that you changed before) to *-1.0*.

Don't forget to change the Z step size back to its original value.

Flipping Traced Data

This method can be applied to one or more individual sections in a data file after tracing is completed.

1. Click **Options>Display Preferences>View** tab and select **Show Current Section Only** and **Show Suppressed as Gray**.
2. Use **Tools>Serial Section Manager** to highlight the section to be flipped. Click on the section to be flipped in the **Section Z** field to make that the active section.
3. Click **Tools>Shrinkage Correction**. In the **Shrinkage Correction** dialog box enter *-1.0* in the **Z** field, and *-1.0* in either the **X** or **Y** field depending on whether you would like to flip the section vertically or horizontally. Entering *-1.0* in the **X** field flips the tracing left to right around the reference point. Entering a *-1.0* in the **Y** field flips the tracing top to bottom around the reference point. If the reference point was not placed in the center of the section, the tracing may be flipped so that it is no longer in the field-of-view. Use **Move>Go To** or the **Go To** command in the **Macro View** window to move the tracing back into the field-of-view, and then use **Joy Free** or **Align** to carefully re-align the section..
4. If the depth values of the flipped data need correcting to match them up with the original section, click **Edit>Select All Objects**, and then right click and select **Modify Z Position** from the menu. In the **Modify Z Position** dialog box, click on **Shift Z Values**, then type in the amount of translation you want to apply to the section in order to correct its position in the serial stack.

Do not use Set Z Values, as this flattens all points in the section to a single Z Value.

5. Use the **Orthogonal View** window to view the relative positions of a series of sections in a serial stack.
6. If you need to rotate or translate this section in X and Y to line it up with the other sections, use **Match Section**. Select both **Display Current Section** and **Show Suppressed as Gray**, then click **Tools>Match Section**. Choose a number of points to align. For each pair, click first on a point on the current (color) section, then click on the location of the gray section where that point should be. After the last pair of points has been placed, the tracing in the active section is re-aligned.

Tools>Match Section can also be used in conjunction with Tools>Where Is to see the entire tracing while doing the match.

7. Save the repaired serial section reconstruction in a newly named file, or if you are confident about the transformation, save it under the original file name.

Chapter 11

The MRI Module

The MRI module is a plug-in for the standard Stereo Investigator program. It is designed to read and write MRI (Magnetic Resonance Imaging) images in the ANALYZE or DICOM file formats.

The MRI Module uses the same commands and procedures as the Image Stack Module. Please see **Image Stack Module**, specifically Loading and Viewing Image Stacks, and Tracing from Image Stacks for more information, commands and procedures.

What file formats are supported?

The MRI Module supports these file formats, which are specific to medical imaging:

- ANALYZE format (.img for image, .hdr for header)
- DICOM format (.dcm)

You can also load image stacks in the Stereo Investigator supported Image Stack file formats. See Supported File Formats.

How do I load and view MRI images?

You use the **File>Image Open** command for DICOM formatted images. Use **File>Image Stack Open** for DICOM or ANALYZE formatted images.

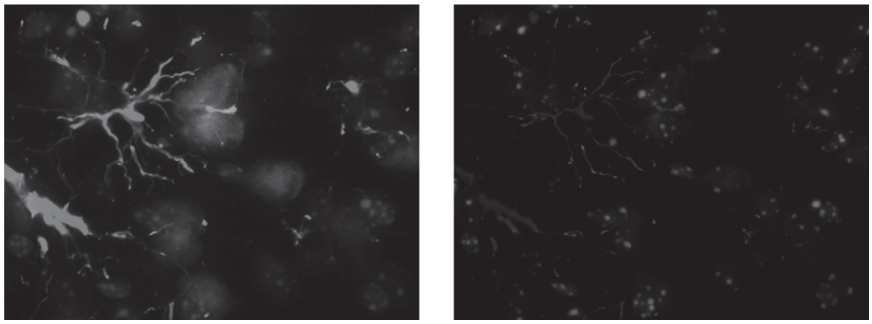
Chapter 12

The Deconvolution Module

MBF Bioscience has partnered with Scientific Volume Imaging B.V. to bring you deconvolution capabilities for your images. This is a separate module you license for use with MBF Software.

What is Deconvolution

Deconvolution reverses the optical distortion that takes place in an optical microscope to create clearer images. It can sharpen images that suffer from fast motion or jiggles during capturing or images that have some type of noise introduced into the signal. Early Hubble Space Telescope images were distorted by a flawed mirror. Deconvolution software corrected for these flaws to produce the correct images. View these images in the Online Help accompanying the software for a better view.



The image on the left shows an uncorrected image with signal noise. Deconvolution was applied to the image on the right. Notice the differences. It is easier to count or measure the structures on the deconvolved images.

What do I need to perform Deconvolution?

You need to have purchased and installed the Huygens Deconvolution module. If you purchase this module after you have already installed Stereo Investigator, contact MBF Bioscience for licensing information.

In addition to the images, you must have the following information about the image and its acquisition:

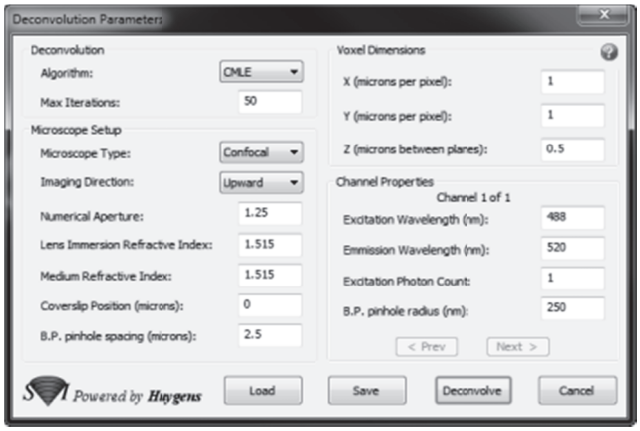
- Microscope type: Confocal, Widefield, or Nipkow disk
- Imaging direction: Upward or downward
- Numerical aperture
- Lens immersion refractive index

- Medium refractive index
- Coverslip position
- BP pinhole spacing

Deconvolving an Image

To deconvolve an image

1. Load an image.
2. Click **Image>Deconvolve Image**. The software displays the **Deconvolution Parameters** dialog box.



3. Select a **Deconvolution Algorithm**. You can choose:
   - **ICTM:** Iterative Constrained Tikhonov Miller
   - **TM:** Tikhonov Miller
   - **QMLE:** Quick Maximum Likelihood Estimate
   - **CMLE:** Classic Maximum Likelihood Estimate
4. Select the **Max Iterations** to perform. (This option is not available if you select the Tikhonov Miller algorithm.)
5. Select your **Microscope Type** and **Imaging Direction**.
6. Type the correct values for the other **Microscope Setup** options:

You must have the correct values. The software cannot detect which values were used. If you do not have these values, deconvolution will not be successful.

   - **Numerical Aperture:** a number describing the amount of light coming from the focus that the objective collects.
   - **Lens Immersion Refractive Index:** Also known as the Lens Refractive Index. This value is the refractive index of the medium the objective is immersed in, such as air, oil, water.
   - **Medium Refractive Index:** This figure is usually the same as that of the **Lens Immersion Refractive Index**. it is the refractive index of the medium the specimen is embeded in.

- **Coverslip Position (microns):** The distance the coverslip is (in Z) from the first focal plane.
- **B.P. pinhole spacing (microns):** The distance between the pinholes on a Nipkow disk. B.P., or backprojected, refers to the size of the pinhole as it appears on the specimen plane.

7. Set the **Voxel Dimensions** in **X**, **Y**, and **Z**. A voxel (volumetric pixel) is the smallest part of a three-dimensional image. The software will read these values from the image file, if present in the file.
8. Set the **Channel Properties** for each image channel.
   - **Excitation Wavelength:** the wavelength of the radiation used to fluoresce the object.
   - **Emission Wavelength:** The radiation emitted back from the object.
   - **Excitation Photon Count:** The number of photons of a given excitation wavelength absorbed by a fluorophore in the object.
   - **B.P. pinhole radius:** The radius of the pinhole as seen from the focal plane. For assistance in calculating this figure, please see the backprojected confocal pinhole calculator page on the Scientific Volume Imaging site.

   Click Prev or Next to move to another channel.
9. Click **Deconvolve**. The software starts the deconvolution process. When complete, the it displays the deconvolved image.

Working with Deconvolution settings

You can save your settings to share or for later use and load other settings.

To load or save settings

1. In the **Deconvolution Parameters** dialog box, click **Load** or **Save**. The software displays a **Load** or **Save As** dialog box.
2. To save, type a name for the file. To load, type the name of the file to load or double-click the name in the dialog box.
3. Click **Open** or **Save** as needed.

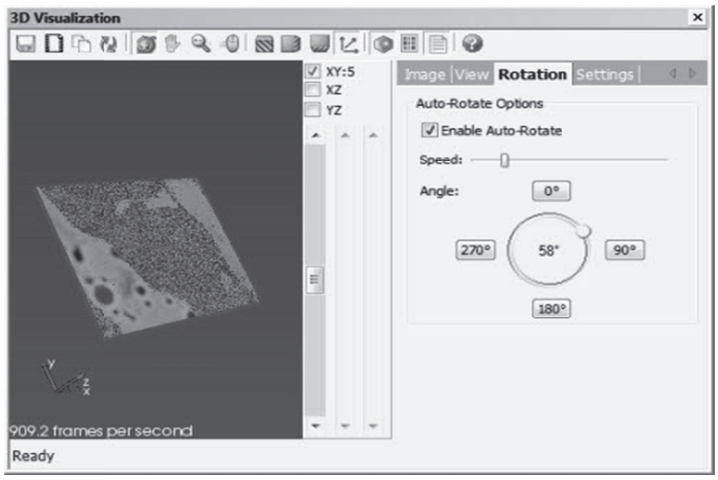
Chapter 13

3D Visualization

3D Visualization replaced the 3D Solids module in previous versions of Stereo Investigator.

The 3D Visualization Interface

The 3D Visualization window is free-floating or dockable, and contains controls you use to change or modify the display.



Working with 3D Objects and Attributes

Within the 3D Solids module, you can change the settings and options for the way contours, trees, and markers are represented in a tracing. You can change Image and Bounding Box options, and you can change 3D depth rendering, autorotation options, and save and manage settings.

Work with image settings

You use the Image tab to work with image, slice, and bounding box settings.

To view the image that you traced, click the **Image** checkbox. To change image options, select the radio button.

You can choose how Stereo Investigator blends the data for your display. your options are:

- **Max Projection:** Choose when the image background is darker than the foreground; typical in fluorescent and confocal images.
- **Min Projection:** Choose when image background is brighter than the foreground; typical in brightfield images.
- **Alpha Composite:** Choose to view different levels of detail. This option also adds 3D depth compared to the previous two projection blend methods.

GPU rendering is available if your graphics card has a separate graphics processing unit. This option directs Stereo Investigator to use the GPU to render the image, resulting in much faster refresh times.

You can adjust **Brightness** and **Contrast** with the sliders.

Transparency makes the image more or less visible against the background or other objects. A higher level makes the image almost invisible while a lower level leaves the image most visible.

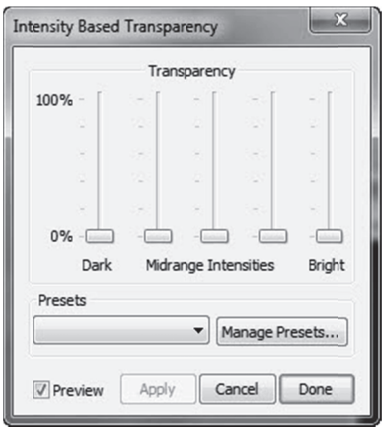
When rendering image data, image properties (luminance, or RGB values) are mapped to optical properties (transparency) while letting the luminance or RGB values go through. Luminance values are fixed as they come from the image. What you are changing is which luminance value is mapped to which transparency value. You are *not* changing the luminance value.

You may find changing the Brightness/Contrast Settings to be a more intuitive way to achieve the desired rendering result of your image data. Nevertheless, you may need to change the luminance to transparency mapping if you need to improve the visibility of other 3D objects intermixed with the image data.

You can work with simple transparency, and adjust the intensity with the slider. You can also use Intensity Based and adjust different transparency aspects.

To change transparency map aspects

1. Click the **Intensity Based** button and then click **Map**. Stereo Investigator displays the **Intensity Based Transparency** dialog box.



2. Adjust the sliders until you are satisfied. The Preview checkbox is selected by default so you can see your adjustments. These are not applied until you click **Apply** or **Done**.

   You can also choose a preset from the **Presets** list.

3. When satisfied, click **Apply** or **Done**.

You can save your new settings in a preset.

1. Click **Manage Presets**.
2. In the **Preset Save/Update** dialog box, type a name for the new view and click **Save**.

To delete a saved preset, select it under **Existing presets** and then click **Delete**.

To save a preset

1. In the **Luminance to Transparency** dialog box, click **Manage Mapping Presets**. Stereo Investigator displays the **Preset Save/Update** dialog box.
2. Type a new name and click **Save**.

Changing Slice Options

Use the sliders to change the brightness and contrast. You can also change the border's display, as well as its color and thickness

Select **Use Linear Interpolation** to "smooth" pixels for display. Your data remains intact, but the display shows your image's pixels with a smoother appearance.

Changing The Bounding Box Options

You can change the thickness and color of the Bounding Box's line.

Use the **Thickness** slider to change thickness.

To change the line color, choose a color from the **Line Color** drop-down.

Work with tracing settings

You can work with contours, trees, and markers settings using the **Tracing** tab.

Contours

To display contours, click the **Contours** checkbox. To work with contour settings, select the radio button under **Configure**.

In the **Contour Options** area, you can display traced contours and how they display. Click the **Traced Contours** checkbox to display traced contours. Select Line to display the contours as lines, or **Flat Surface** to display them as flat surfaces with no depth.

Click **Surface Reconstruction** to see the surface. Choose **Solid** to put a solid skin over the contours. Choose **Wireframe** to display the surface as a wireframe image.

This wireframe image is triangulated and different than that of the individual contours displayed as lines.

To close any open contours, choose **Cap Ends**.

To round edges, choose Smooth. Use the slider to control the amount of smoothing applied.

You can control the contour transparency with the Transparency control. Move the slider right for more transparency. You can choose to apply this setting to all contours, or choose a contour from the drop-down list.

Trees

To display trees, click the **Trees** checkbox. To work with trees settings, select the radio button under **Configure**.

Click **Traced Centerlines** to show the centerlines of the trees.

To see the rendered surface of the trees, click **Surface Reconstruction**. You can view the surface reconstruction as a solid or a wireframe. Select the desired radio button.

Click **Smooth** and use the slider to view and adjust the smoothness of the reconstruction.

Use the Transparency slider to adjust the tree transparency.

Markers

If you placed markers, you can also modify their display in 3D solids.

To display markers, click the **Markers** checkbox. To work with marker settings, select the radio button under **Configure**.

Click **Symbol** to display the marker symbol; click **Outline** to display the marker as an outline.

Use the **Transparency** slider to adjust the tree transparency.

Work with view settings

You use the view settings tab to select and set up view angles. We include six pre-defined view angles—front, left, top, back, right, and bottom. Click a button to switch to that angle.

If you have any saved view angles, select it from the drop-down list and click the **Saved** button.

You can click **Zoom to Fit** to have Stereo Investigator adjust the zoom to fit the entire image and tracing in the window.

Saving And Organizing Views

You can save views to use again and delete views you no longer need.

1. Click **Save / Organize**.
2. In the **Preset Save/Update** dialog box, type a name for the new view and click **Save**.

To delete a saved view, select it under **Existing presets** and then click **Delete**.

Work with rotation settings

This tab controls rotation. You can set speed and angle options for **Auto-Rotate**.

Use the **Speed** slider to set the autorotation speed.

Use the preset buttons to choose an angle(0, 90, 180, or 270) for autorotation. You can also use the dial control to have a finer control over the angle.

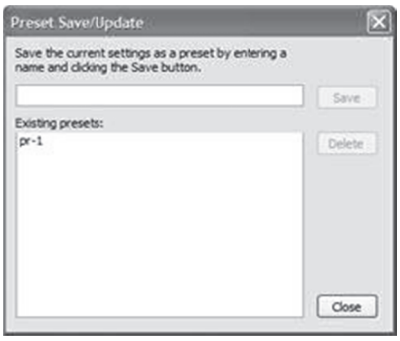
You can save your settings and edit previously saved settings with the **Settings** tab, as well as set rendering options and background color.

To load a settings file

- Choose a settings file from the **Saved** drop-down.

To save a new settings file

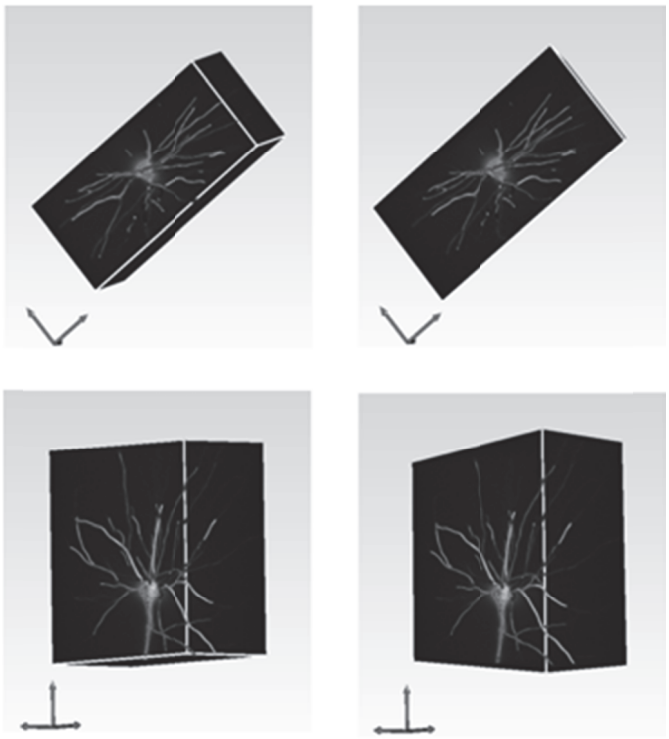
1. Under **Image and Tracing Settings**, click **Manage**. Stereo Investigator displays the **Preset Save/Update** dialog box.



2. Type a name for the preset, and then click **Save**.

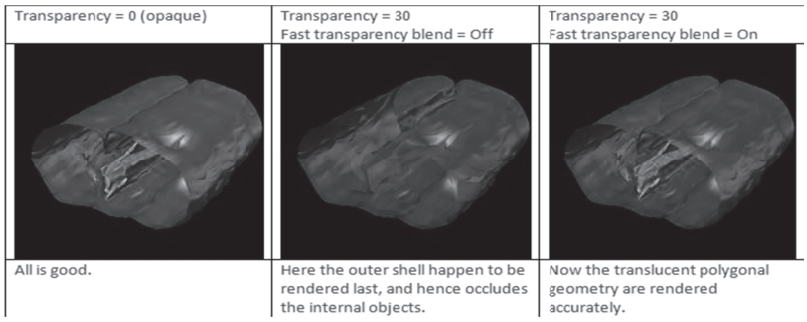
Rendering Options

- **Use perspective projection.** Perspective displays objects (lines, planes) that converge to a vanishing point. The easiest way to understand using this feature is to mouse over the following graphic.



Images in the left column have User perspective projection off; those in the right column have User perspective projection on. When off, the image looks unnatural or distorted from any angle other than the front or side view. Normally, User perspective projection is active. However, for a more accurate front or side view, turn this option off.

- Notice the images on the left, with perspective projection turned off and compare them with the images on the right with perspective projection turned on. When perspective projection is off, the image looks unnatural or distorted from any angle other that front or side view. Normally, **Use perspective projection** is active. However, for a more accurate front or side view, turn this option off.
- Fast transparency blend renders translucent overlapping objects in no particular order. Turning triggers an algorithm that renders translucent objects from front to back until it detects no more objects to render. This takes more computation, but results in accurate transparency blend.



Translucent here means somewhat transparent, i.e. not opaque, transparency > 0. Here, objects only include the elements of the tracing model. Image stacks are not affected.

- **Display frame rate** lets you set the color of the text that updates frame rate information at the bottom of the **3D Visualization** window.

Background Color

You can set the background color with the color picker. You can also set a "top" color that blends into the background color with the **Top** control.

Chapter 14

The Image Montage Module

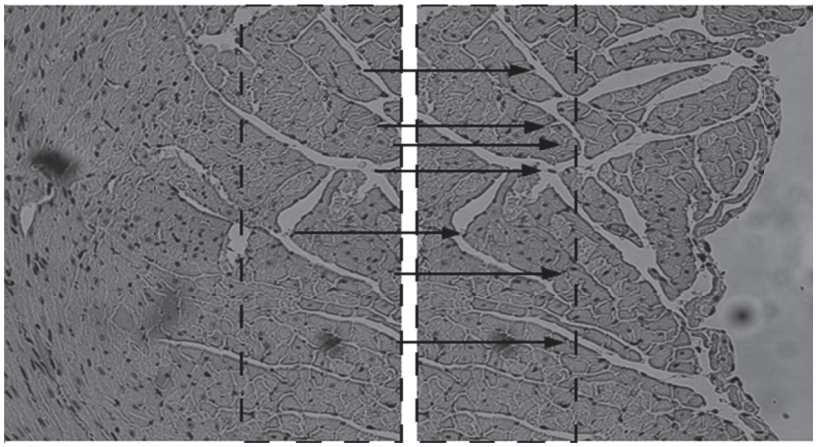
The Image Montage module is an extension to the standard version of MBF Bioscience software, providing an additional capability of creating 2D and 3d image montages from images and image stacks.

The Image Montage module allows you to load a collection of images or image stacks and manually or automatically organize them into a montage. In automatic mode, the Image Montage command arranges the individual images with correct X and Y (and for stacks, Z) alignment. You can then save the entire image or save the DAT file. There is no limit to the number of images that you can montage, except as constrained by your computer memory.

Image montaging and how it works

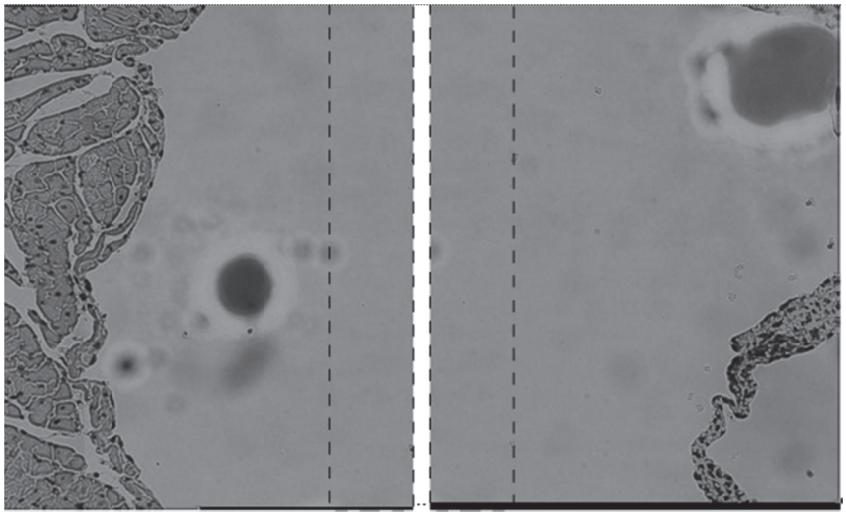
We use sophisticated algorithms to perform automatic image montaging. How it detects and matches features (or points) in images determines how well automatic montaging occurs. Here are some general guidelines and answers to some questions you might have. Click a heading below for information.

Automatic montaging matches on features, or points. We define a feature as a point, for which there are two dominant and different edge directions in a local neighborhood of the points. To match two images, we need enough features—or points—in the overlapping region of the two images. In this figure, montaging was successful because the algorithm found enough points (shown as green point) inside the overlapping region (the area between the dashed lines).



The arrows show where the algorithm matched the points.

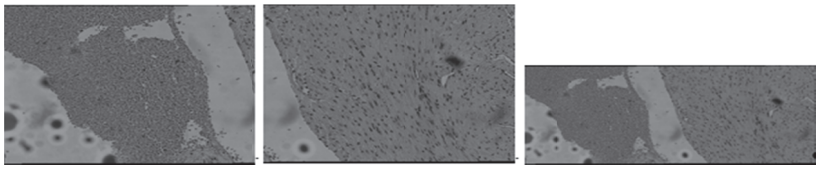
In the next image, however, automatic montaging failed because it found too few matching points within the region of overlap.



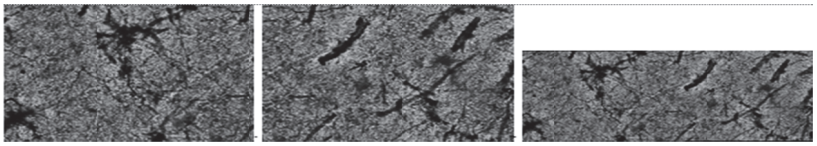
Without these points, it couldn't complete the montage.

The next factor that affects automatic montaging is the amount of overlap (overlapping pixels) between images. As a rule of thumb, the larger the overlapping region, the better performance for automatic montaging. However, the larger the overlapping regions, the more computational time is required to perform automatic montaging.

From our experience, an overlapping ration of between 10% to 25% of the image sizes works best, and is recommended to start. In the following image, we used a ratio of 12.5% of the first two images, which produced a good montage in the final image.



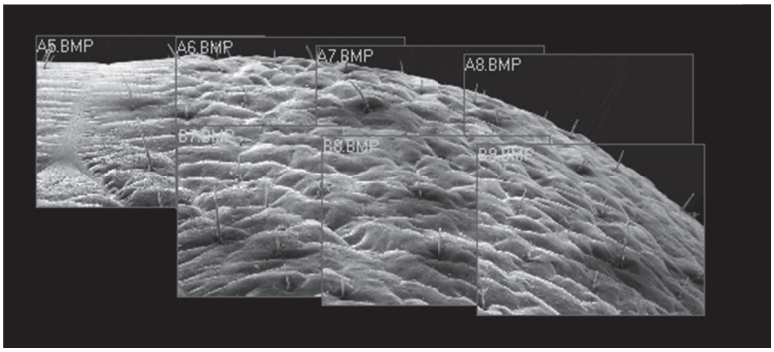
In the next set of images, though, we needed to use an overlapping ratio of 25% for a successful montage.



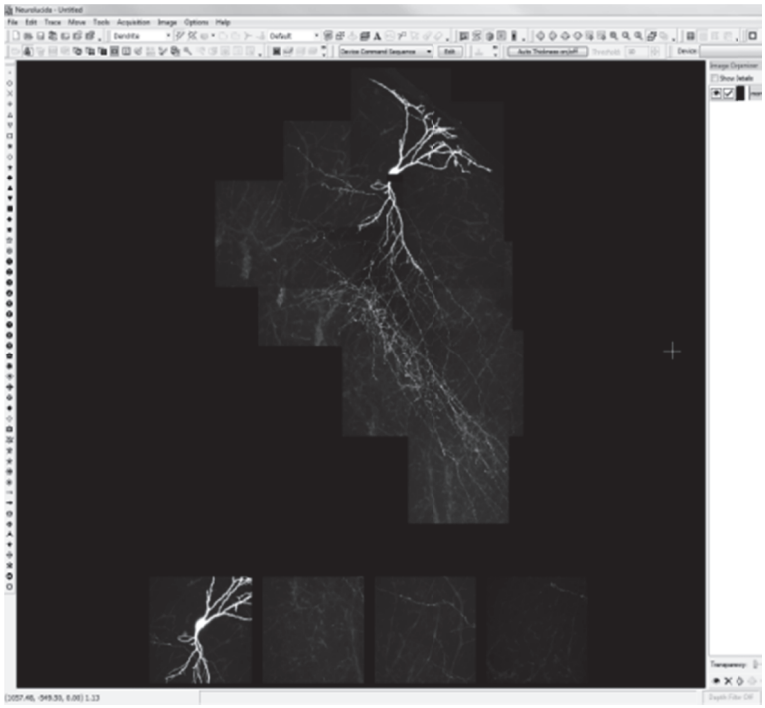
By selecting a proper overlapping region and overlapping ratio, you should end up with a complete image montage. Sometimes this doesn't happen, however, and you are left with extra images that aren't included in the montage. Using an improper overlapping region or using an overlapping ratio too large or too small can cause some images to be orphaned. Image Montage moves these orphaned images below those that it can align. Try using images with a different overlapping ratio or different regions.

Another reason for orphaned images is that they are unrelated to the other images used in the montage. This can occur if you've saved images to the wrong place or if the images are from the same sample but have no related features. In that case, automatic montaging only uses the images it can match, and it moves the ones it can't match below those it can. .

Sometimes the images can't be automatically aligned in XY because of an inherent property of the image. Notice this montage of an insect eye. If you look closely, you'll see that the images don't align exactly. They almost look like they are tilting away from each other.



We can also see failed Z alignment in this image.



Even with failed automatic alignment, however, you can manually work with the images to get a closer alignment and a better montage.

The software needs information from the XY alignment before it can perform the Z alignment. We allow alignment in XY, and also XYZ, but the Z alignment happens internally after doing the XY alignment. It can't align just Z without knowing where it is in XY.

What file formats are supported?

We support the following formats:

MBF JPEG2000 (.jp2; .jpx; .jpf)	MBF Tiff (.tif; .tiff)	JPEG2000 (.jp2; .jpx; .jpf)
Tiff (.tif; .tiff)	Bit Map (.bmp)	JPEG (.jpg, .jpeg)

ZSoft (.pcx)	PNG files (.png)	TARGA files (.tga)
Olympus Fluoview (.tif)	Portable Image (.pgm; .pbm; .ppm)	BioRad Confocal Image (.pic)
FlashPix (.fpx)	Zeiss Confocal LSM (.lsm)	Zoomify (.pff)
DICOM (.dcm)	ANALYZE (.img)	NanoZoomer (.ndpi; .vms; .vmu)
Aperio SVS (.svs)		

Loading images and image stacks

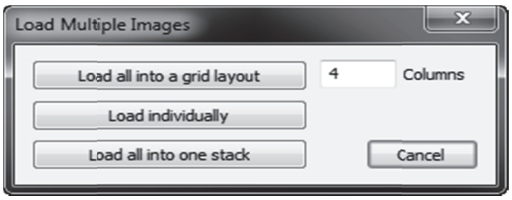
You can load images with the **File>Image Open** command or with drag and drop.

To open a set of images

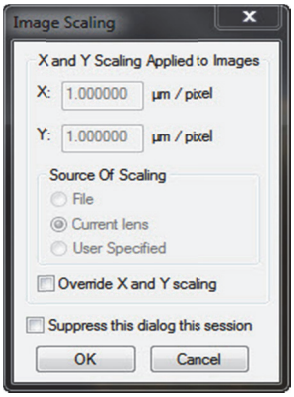
1. Click **File>Images for Montage Open**. Stereo Investigator displays the **Image Open** dialog box.
2. Click a file name. Hold down **CTRL** to select non-contiguous files. Hold down the **SHIFT** key to select contiguous files.
3. Click **Open**. Stereo Investigator loads the files into your workspace.

To drag a set of images

1. In Windows, open the folder containing your files.
2. Select the files by click-dragging, or click a file and then hold down **CTRL** to select non-contiguous files or hold down the **SHIFT** key to select contiguous files.
3. Drag the files to the workspace. Stereo Investigator displays the **Load Multiple Images** dialog box.



4. Choose a loading option
   - Load all into a grid layout: Loads the images into a columnar grid.
   - Load individually: Loads each image individually. Use this option if you need to set individual scaling for each image.
   - Load all into one stack: Loads all the images into one image stack
5. Stereo Investigator displays the **Image Scaling** dialog box.



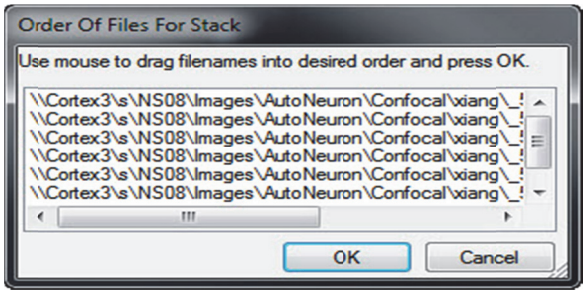
Stereo Investigator needs this information if it is not contained in the image file.

6. Click **OK**. Stereo Investigator loads the files into a grid. If you chose to load each image individually, Stereo Investigator displays the Image Scaling dialog box for each image.

If the image files don't contain scaling information,

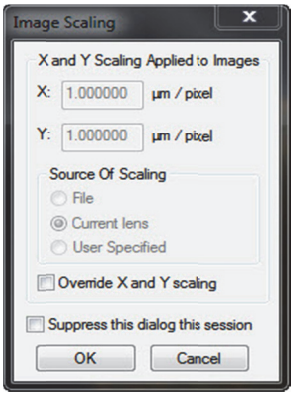
To open an image stack composed of several files for a montage

1. Click **File>Image Stack Open**. Stereo Investigator displays the **Image Stack Open** dialog box.
2. Select the images and click Open. Stereo Investigator displays the Order of Files for Stack dialog box.



If the files are not in the proper order, you can drag them in the list until the order is correct.

3. Stereo Investigator displays the **Image Scaling** dialog box for X and Y scaling. Stereo Investigator needs this information if it is not contained in the image file.



4. Click **OK**. Stereo Investigator loads the files into a grid. If you chose to load each image individually, Stereo Investigator displays the Image Scaling dialog box for each image.
5. Click **OK**. Stereo Investigator displays the **Image Scaling** dialog box for Z scaling.

Since single image files do not contain Z spacing information, you need to manually enter this information. The program prompts you to enter the image separation while loading the stack. This is the distance between images. You can use the focal distance or the physical distance.

- **Focal Distance**—Image stacks collected with Stereo Investigator are collected using spacings that describe the focal plane separation.
- **Physical Distance**—Describes the physical movement of the microscope stage as images are collected. If you select this option, correction factors must be applied to convert the microscope movement into the movement of the focal plane. The X and Y dimensions of the imported image default to the micron/pixel ration for the current lens. Select the lens that was used to capture the images before loading the image stack. If the image stack was collected on a different microscope it is important to calibrate a lens for that system. Select that lens before loading the image stack.
- If you select this option, you need to select the correction factor for the physical distance between the lens and the image. Stereo Investigator automatically enters this value for **Air, Oil,** and **Water**. If you select **Other,** you must manual enter the factor.

6. You can use the X and Y scaling used when the image was acquired, or override it. Click **Override X and Y scaling**, choose the source, and then enter the values.
7. Click **OK**. Stereo Investigator loads the image stack as a montage.

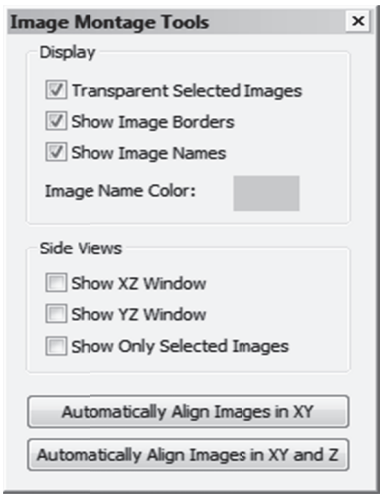
If you load an image for which there is no matching lens, Stereo Investigator prompts you to define a new lens to match the image scaling. For information on defining a lens, see Defining lenses.

Creating image montages

Once loaded, you use the Image Montage command to display and align the images.

To use image montage

- Click **Image>Image Montage**. Stereo Investigator displays the **Image Montage Tools** dialog, a dockable window.



You use this dialog to control **Display** options to help you move and align images.

- **Transparent Selected Images:** Makes the images transparent. This is useful when manually aligning the images, making it easier to see structures and landmarks in the images.
- **Show Image Borders:** Displays a fine line border around each image.
- **Show Image Names:** Displays the image name in the top left corner of each image.
- **Image Name Color:** Lets you use the color picker to change the color of the displayed image name.

Because the Image Montaging algorithm needs image features to accurately and precisely align the images, other Image menu commands such as **Maximum Intensity Projection** or **Minimum Intensity Projection** can be used to create a more precise image montage.

Side View options let you display different views of the images.

- **Show XZ window:** Displays a separate dockable window showing the XZ projection.
- **Show YZ Window:** Displays a separate dockable window showing the YZ projection.
- **Show Only Selected Images:** Displays only the images you have selected. You can click on images to select and deselect them. Use the SHIFT key to select to select more than one image. You can also click-drag a marquee around images to select them.

If you make a mistake, the **Undo** command works on many Image Montage actions.

Automatic Alignment

Two buttons control automatic alignment:

- Automatically Align Images in XY
- Automatically Align Images in XY and Z

Click a button to begin the alignment. Depending on the number of images and their complexity, automatic alignment can take some time. The progress indicator shows the approximate time remaining.

When complete, you should examine the montage. If you are using an image stack, use the **Page Up** and **Page Down** keys to travel up and down the Z axis when checking alignment. You can also use the Cursor/arrow keys to align.

Manual Alignment

We suggest using automatic alignment as a first step. When automatic alignment completes, you can use manual alignment to fine tune the alignment if it is off. Click-drag an image to move it. You can select multiple images using the SHIFT or CTRL keys.

Saving image montages

You can save image montages as images or as DAT files. When you save a montage as an image, you "flatten" all the images into one image—you can't manually move or realign the individual images after saving as a new image. When you save as a DAT file, you save the files and layout structure so that you can later open the DAT file and work with the image montage and its tiles.

To save image montages

- Click **File>Save Data File** or **File>Save Data File** As to save as a data file.
  -or-
  Click **File>Montage Save As** to save the new montaged image.

Chapter 15

The Virtual Tissue Module

The Virtual Tissue module is an extension of the standard MBF Bioscience software that provides you with the additional capability of creating extremely high-resolution montages composed of images obtained from multiple microscopic fields of view. The Virtual Tissue module uses a motorized stage to automatically collect a series of contiguous images of a specimen and merge them into a single image montage, which we refer to as a Virtual Tissue Image, or "virtual slide".

The Virtual Tissue tools are only accessible if you have purchased the Virtual Tissue module.

Uses for Virtual Tissue

- Virtual Tissue allows you to view an entire specimen in one field-of-view, as if you had an ultra-low power objective lens.
- Virtual Tissue can help you to prepare image montages for publication.
- Virtual Tissue can generate a true Macro View that can be used to navigate through the tissue section.
- Montage images can be used with any Stereo Investigator analysis tools, making it possible to work away from the microscope.
- Images can be saved in multiple formats to optimize size and resolution for printing or publication.
- Virtual Tissues can be saved into a custom web-based database for viewing over the Internet. (3D tissue is limited to JPEG200 formatted files.)

Setting Up for a Virtual Tissue Acquire

Once the initial parameters for image collection have been set, Virtual Tissue acquisition proceeds automatically, driven by the software. If you are not pleased with the results , take a moment to fine tune the collection parameters. Feel free to contact us for help with image optimization.

Check your Calibration and Alignment: The Virtual Tissue acquisition depends on good calibration and alignment for seamless stitching of the image tiles. Please see Defining And Calibrating A New Lens for ways to quickly check and correct the lens calibration and camera alignment.

Find the area of interest on the slide: If you would like the entire area or section to be acquired, the best way to proceed is to trace a contour around the area of interest (or the whole section) at low magnification. The contour does not need to be precise, and is not included in the virtual slide, but is a convenient way to let the software know what should be included in the montage. Once the contour is

traced, remember to use Joy Track for any joystick movement so that alignment between the tracing and specimen is retained.

Choose a Magnification: Choose a magnification that gives you a sufficiently detailed field-of-view. Remember that choosing too high a magnification results in virtual slides that take up very large amounts of disk space. Choosing too low a magnification results in images that do not have sufficient resolution of detail.

Prepare the microscope: Set the field aperture, neutral density filters, condenser height, condenser aperture, and camera settings to provide even illumination across the entire field-of-view. If the PreFocus mode is not being used, adjust the slide holder so that as the slide moves in the XY plane it remains in proper focus.

Adjust the camera: Set the camera to manual shutter mode and manual gain mode so that all images are captured at the same exposure. For color cameras set the white balance before acquiring a virtual slide. For black and white cameras, you may also want to set the black balance.

Be sure the image is optimized: Look at Image Adjustment (Imaging>Image Adjustment) for each color channel to make sure none of the channels are saturated. If so, adjust the camera settings so that none of the channels are saturated.

Set and Enable Background Correction: Focus on the specimen, adjust the light, condenser, etc., for an optimum image. Next, move the stage to view a portion of the slide containing no tissue or dust particles. (Use Joy Track or the Field Move commands to make it easy to return to your tissue specimen.) Do not change any of the camera settings. Click **Acquisition>Acquire Background Image**, and then check **Enable Background Correction**. To view the background correction image, select **Acquisition>Display Background Image** or select the **Display Background Image** button.

If you need to reset the background correction, or are starting a new image, acquire another background image.

Select **Imaging>Live Image**.

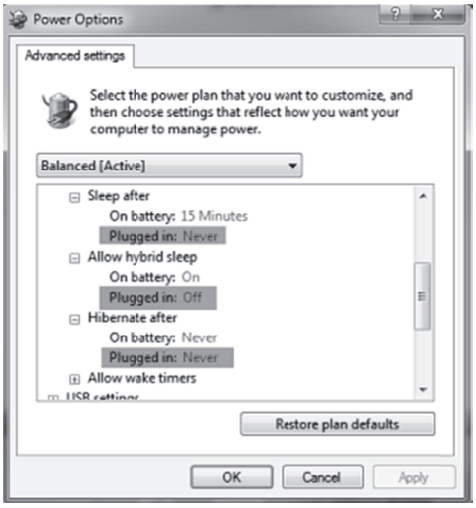
Move the stage back to the specimen.

If you are acquiring overnight or over a long period of time

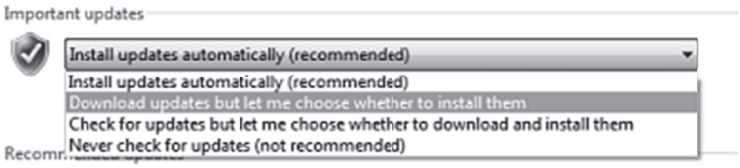
If you are performing a long acquisition, you need to take some steps to assure success. Before staring, you must prevent the computer from entering sleep mode and you must turn off automatic install of updates.

To set for a long acquire

1. Click **Control Panel>Power Options** and then click **Change advanced power settings**.
2. Set your power options as shown here.



3. Click **OK** to apply and close this dialog.
4. In **Control Panel**, click **Windows Update**, and then click **Change settings**.
5. Change to the second option in the list, as seen here.



6. Click **OK** to apply the change and close the dialog.
7. Exit from **Control Panel** and continue with your work.

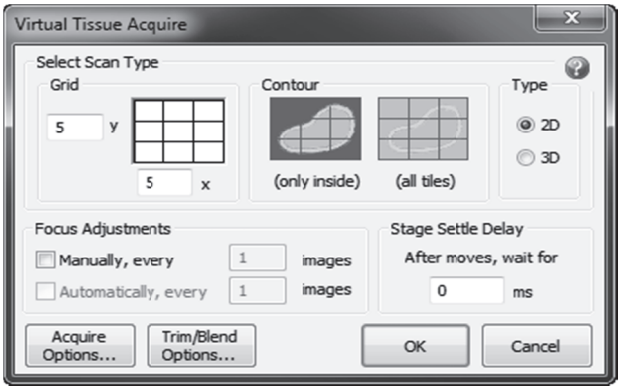
First Steps to Acquire

The best way to proceed is to run a trial acquisition of a small number of fields of view in order to fine tune the collection and merging parameters for your system before acquiring a larger image.

You can acquire a 2D (x, y) image or a 3D (x, y, z) image.

To start a Virtual Tissue acquire for a 2D image

1. Click **Acquisition>Acquire Virtual Tissue**, or click the **Virtual Tissue** toolbar button. Stereo Investigator displays the **Virtual Tissue Acquire** dialog box.



2. Select **2D** under **Type**.
3. Set your options (described below) and continue with **To perform Virtual Tissue Pre Focus**.

Acquisition Options

There are two methods for acquiring virtual slides, depending on how you choose to define your area of interest.

Grid Scan: Choose Grid Scan if you want to define the area to be acquired as a set number of fields of view in a rectangular grid. This option requires that you be positioned at the top left corner of the region to be scanned. The current field-of-view is the first one scanned

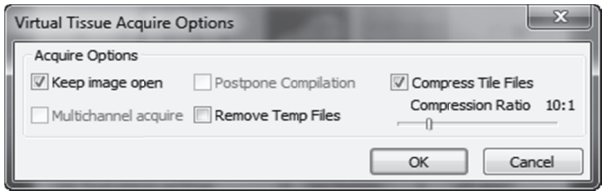
Before starting a virtual slide acquisition acquire a small test image to fine tune the collection settings. Select Grid Scan, and enter a 3×3 scan in the first panel of the dialog box. Use this trial scan to adjust the settings discussed below. Start with stitching and blending disabled and adjust the overlap and trimming as necessary. Stitching and blending can be enabled later if necessary.

Place the reference point at the top left corner of the first acquisition field of view, then to return to the beginning of the scan, use **Move>To Reference Point**. Choose an easily identified point not far from the area you will be studying so that you can return to it without difficulty. If you are working with serial sections, the reference point is best located near the initial section of the series.

Contour Scan: You must draw a closed contour around the region to be scanned for a Contour Scan. There are two options for determining the fields to be included in a Contour Scan:

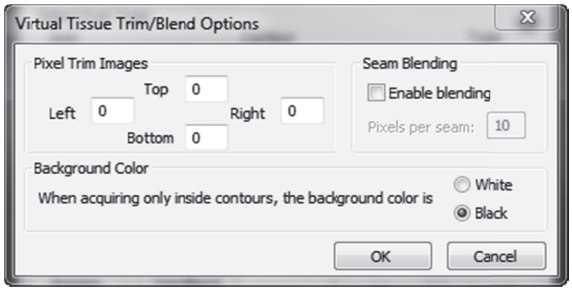
- **Only inside:** Every field that has any portion within the contour will be acquired and included in the final virtual tissue output. This options saves considerable memory space if the object is not approximately rectangular.
- **All tiles:** A rectangle is generated that contains the entirety of the contour. All fields within this rectangle are scanned and included in the final virtual tissue output. This option generates a rectangular virtual tissue that may have many fields that do not include the outlined specimen.

Acquire Options: This button displays the **Virtual Tissue Acquire Options** dialog box containing the following options:



- **Keep image open:** Opens the image when the acquire is complete..
- **Multichannel acquire:** Uses the existing Multichannel acquire setup. You should change this setup prior to performing a Virtual Tissue acquire.
- **Postpone Compilation:** Postpones the compilation. It can be done later on another machine with the Virtual Tissue Compiler command.
- **Remove Temp Files:** Removes the temp files after compilation.
- **Compress Tile Files:** Compresses the tiles by the specified ratio. Use the slider to change the ratio. A 20:1 ratio is considered safe.

Trim / Blend Options displays the Virtual Tissue Trim/Blend Options dialog box used to set the trimming, blending, and background color options.



Pixel Trim: This option removes rows and columns of pixels from the edges of each field-of-view (image tile), thus removing information. This option allows you to correct for bad spherical aberration, or a video card that acquires with a black or white strip on any of the image edges. Try a trial acquisition with all of these values set to zero, and increase them if there is a problem in the final image. Acquire a single image, then zoom in on the top left and bottom right corners. Any rows or columns of bad pixels are immediately evident. Set the trim large enough to remove these pixels.

Sometimes if lighting is very uneven across the field of view, large numbers of pixels need to be removed to account for unevenly lit portions of the image. When many pixels are trimmed, the fields of view in the resulting virtual slice are made smaller, and smaller stage movements are made accordingly. A lot of pixel trim will slightly increase your acquisition time, since more acquisition sites are required.

Seam Blending: This option smoothly blends the edges from each field-of-view. Use the 3x3 trial to see if your image looks acceptable without blending, as blending involves a slight loss of image detail. We recommend first trying to improve the alignment of tiles by improving the system calibration and alignment or by using Seam Alignment, as Seam Blending does not move the tiles, only blurs the edges to make the seams less distinct. Select the number of pixels to blend at each seam. Generally 5-10 pixels is sufficient.

2D Only

Focus Adjustments: You can set the acquire to prompt to ask you to manually adjust after a predetermined interval of images or tell the software to automatically make focus adjustments.

Note: Automatic focus adjustments requires special hardware. If this option is gray, the hardware is not installed or connected.

Stage Settle Delay: If you are collecting an image with brightfield illumination, the time delay is used to allow time for the stage to stop moving and any vibration to die down. For your trial montage, set this

value to zero, and increase it if you see signs that the stage is still moving when the image is captured. If there are artifacts with a zero time delay, 100ms is usually sufficient delay. However, with some of the slower stages (especially Prior stages), delays of up to 1000 ms may be needed. If you are acquiring fluorescent or other low light images that requiring integration by the frame grabber, enter a value larger than the time of the integration (This may need to be as much as two times the integration time, but start with a number only slightly larger, and increase if you see text in some individual tiles). The software does not automatically know how long the acquisition is, so be sure to allow time for the frame grabber integration.

If you see text in the individual image tiles when using an Optronics camera, pushing the Exposure button on the front panel of the camera controller will make the text disappear.

3D Only

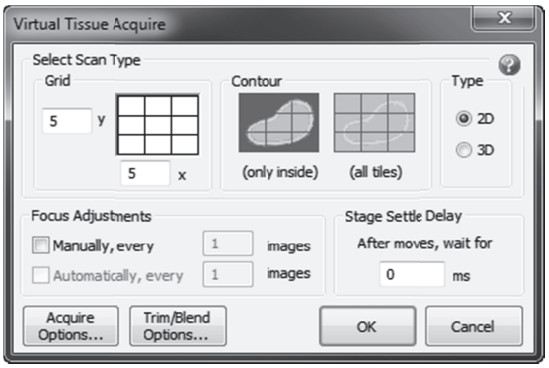
Stack Options include:

- Use Z at time of acquire or Set Top a number of focal planes: You can use the Z value at the time of the acquire (default) or set a number of focal planes to use for the top.
- Use focal planes settings or Set Bottom a measurement of micros apart: Use the focal plane settings or set the bottom

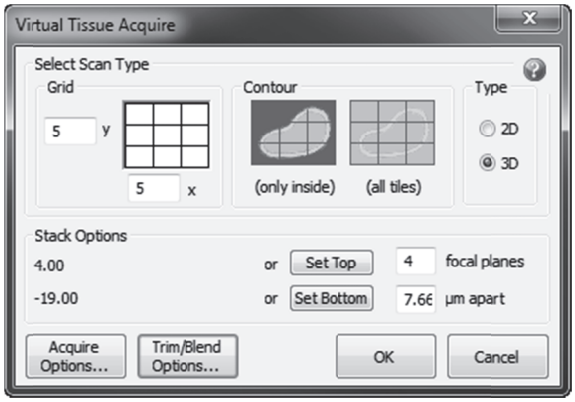
The number of focal planes is the number of slices that will be acquired. If you choose the **Set Top / Set Bottom** options, use the mouse wheel to set the Z positions for each measurement.

To start a Virtual Tissue acquire for a 3D image

1. Click **Acquisition>Acquire Virtual Tissue**, or click the **Virtual Tissue** toolbar button. Stereo Investigator displays the **Virtual Tissue Acquire** dialog box.



Select **3D** under **Type**. The dialog box changes to display 3D options.



2. Set you options (described below) and continue with **To perform Virtual Tissue pre focus**.

Acquisition Options

There are two methods for acquiring virtual slides, depending on how you choose to define your area of interest.

Grid Scan: Choose Grid Scan if you want to define the area to be acquired as a set number of fields of view in a rectangular grid. This option requires that you be positioned at the top left corner of the region to be scanned. The current field-of-view is the first one scanned.

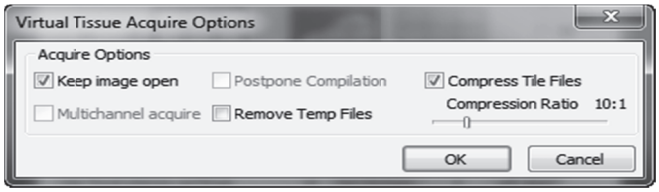
Before starting a virtual slide acquisition acquire a small test image to fine tune the collection settings. Select Grid Scan, and enter a 3×3 scan in the first panel of the dialog box. Use this trial scan to adjust the settings discussed below. Start with stitching and blending disabled and adjust the overlap and trimming as necessary. Stitching and blending can be enabled later if necessary.

Place the reference point at the top left corner of the first acquisition field of view, then to return to the beginning of the scan, use **Move>To Reference Point**. Choose an easily identified point not far from the area you will be studying so that you can return to it without difficulty. If you are working with serial sections, the reference point is best located near the initial section of the series.

Contour Scan: You must draw a closed contour around the region to be scanned is required for a Contour Scan. There are two options for determining the fields to be included in a Contour Scan:

- **Only inside:** Every field that has any portion within the contour will be acquired and included in the final virtual tissue output. This options saves considerable memory space if the object is not approximately rectangular.
- **Acquire tiles:** A rectangle is generated that contains the entirety of the contour. All fields within this rectangle are scanned and included in the final virtual tissue output. This option generates a rectangular virtual tissue that may have many fields that do not include the outlined specimen.

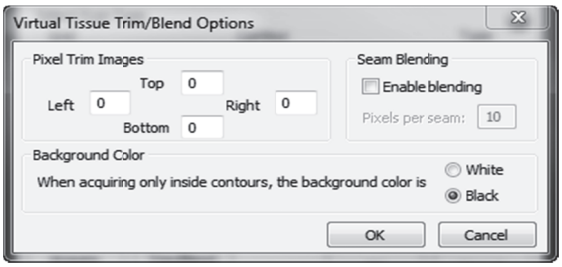
Acquire Options: This button displays the **Virtual Tissue Acquire Options** dialog box containing the following options:



Keep image open: Keeps the image open during the acquire.

- **Multichannel acquire:** Uses the existing Multichannel acquire setup. You should change this setup prior to performing a Virtual Tissue acquire.
- **Postpone Compilation:** Postpones the compilation until
- **Remove Temp Files:** Removes the temp files after compilation.
- **Compress Tile Files:** Compresses the tiles by the specified ration. Use the slider to change the ration. A 20:1 ratio is considered safe.

Trim / Blend Options displays the Virtual Tissue Trim/Blend Options dialog box used to set the trimming, blending, and background color options.



Pixel Trim: This option removes rows and columns of pixels from the edges of each field-of-view (image tile), thus removing information. This option allows you to correct for bad spherical aberration, or a video card that acquires with a black or white strip on any of the image edges. Try a trial acquisition with all of these values set to zero, and increase them if there is a problem in the final image. Acquire a single image, then zoom in on the top left and bottom right corners. Any rows or columns of bad pixels are immediately evident. Set the trim large enough to remove these pixels.

Sometimes if lighting is very uneven across the field of view, large numbers of pixels need to be removed to account for unevenly lit portions of the image. When many pixels are trimmed, the fields of view in the resulting virtual slice are made smaller, and smaller stage movements are made accordingly. A lot of pixel trim will slightly increase your acquisition time, since more acquisition sites are required.

Seam Blending: This option smoothly blends the edges from each field-of-view. Use the 3x3 trial to see if your image looks acceptable without blending, as blending involves a slight loss of image detail. We recommend first trying to improve the alignment of tiles by improving the system calibration and alignment or by using Seam Alignment, as Seam Blending does not move the tiles, only blurs the edges to make the seams less distinct. Select the number of pixels to blend at each seam. Generally 5-10 pixels is sufficient.

2D ONLY

Focus Adjustments: You can set to ask you to manually adjust after a predetermined number of images or tell the software to automatically make focus adjustments.

Stage Settle Delay: If you are collecting an image with brightfield illumination, the time delay is used to allow time for the stage to stop moving and any vibration to die down. For your trial montage, set this value to zero, and increase it if you see signs that the stage is still moving when the image is captured. If there are artifacts with a zero time delay, 100ms is usually sufficient delay. However, with some of the slower stages (especially Prior stages), delays of up to 1000 ms may be needed. If you are acquiring fluorescent or other low light images that requiring integration by the frame grabber, enter a value larger

than the time of the integration (This may need to be as much as two times the integration time, but start with a number only slightly larger, and increase if you see text in some individual tiles). The software does not automatically know how long the acquisition is, so be sure to allow time for the frame grabber integration.

If you see text in the individual image tiles when using an Optronics camera, pushing the Exposure button on the front panel of the camera controller will make the text disappear.

3D Only

Stack Options

First set the number of focal planes, and use the mouse wheel to set the top Z position and click **Set Top**. Then type the measurement in micros these focal planes are apart. using the mouse wheel again, set the bottom Z position. then click **Set Bottom**.

The number of focal planes is the number of slices that will be acquired, based on your measurements. .

To perform Virtual Tissue prefocus

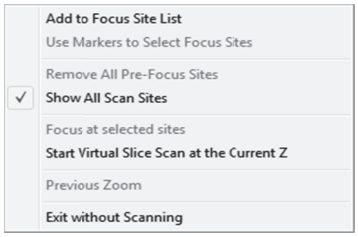
If you haven't set your Virtual Tissue options, please see **To start a Virtual Tissue acquire for a 2D or 3D image**, above.

- After you set the options for a Virtual Tissue acquire and click OK in the **Virtual Tissue Acquire** dialog box, Stereo Investigator displays the grid over the tissue based on the Z-depth at which the tissue is in focus at a user-specified number of individual sites. The calculated focal plane has an orientation based on the manual determination of best focus at least three points on the tissue. Before starting the acquisition, be sure the image is optimized and background correction set by following the directions in See Acquiring Virtual Tissues: Set-up above.

Selection Of Prefocus Sites

If multiple contours are contained in the file, an overview of the entire file is shown, and the status bar contains instructions to left click in the contour to be scanned.

The overview of the selected contour is shown with grid lines indicating the individual fields-of-view to be visited during the virtual slice acquisition. In this mode, you can use these right-click options.



:

Add to Focus Site List: To select sites where the focus is recorded (the Pre-Focus sites), right click over a given site and choose this option. All currently selected Pre-Focus sites are displayed with a highlighted outline.

To change the displayed sites

- Right click on a site to be removed from the list and select **Remove from Focus Site List**.

- or-

- Right click anywhere and select **Remove All Pre-Focus Sites**.

Use Markers to Select Focus Sites: Place markers to mark and select sites for focus.

Show All Scan Sites: Displays the grid.

2. To begin the virtual slice acquisition, right click over the preview and select **Focus at Selected Sites**. Stereo Investigator displays the **Focus Sites** dialog box.

3. Focus at the desired focal plane an click **OK**. The software moves you to the next scan site.
   If you choose **Skip this Site**, the Z position isn't recorded.
   Choose **More** to display the **Device Control** dialog box, which you use to change multichannel setup, camera settings, and devices.

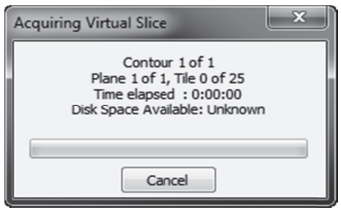
4. Continue focusing at each site. When complete, Stereo Investigator displays the grid with the Z positions shown.

With pre focus complete, you can move on to acquiring virtual tissue.

Acquire Virtual Tissue

To acquire the Virtual Tissue

1. Right-click over the preview and select **Start Virtual Slice Scan with Prefocus** (if you performed a prefocus on sites) or **Start Virtual Slice Scan at Current Z**. The software displays the **Acquiring Virtual Slice** dialog box, and the **Manual Focus** dialog box, when appropriate



2. You can **Skip** focusing on a site, click **OK** to focus on it, click **More** to control the camera and devices, or click **Cancel** to cancel focusing.
3. Stereo Investigator visits each site for you to focus. Once all sites have been visited and a Z-depth recorded, the orientation of the virtual plane is calculated, and the Virtual Tissue begins.

Acquisition Tips: Minimize vibration and light changes in the room used for acquisition. For best results, close the door and keep the lights low during acquisition, and avoid unnecessary walking near the microscope.

After the scanning of sites has completed, the software compiles the tiles into an image. You can compile later if you chose **Postpone Compilation** in the **Virtual Tissue Acquire Options** dialog box.

When acquiring a Virtual Tissue, we display a "pseudo-live" display, the last live image before the acquire starts.

The Virtual Tissue Compiler

You use the **Virtual Tissue Compiler** command to create a Virtual Tissue image from existing acquire virtual tissue acquires. You must have the virtual tissue acquire files and the associated AssemblyData.txt file.

To compile virtual tissue files

1. Click **Acquisition>Virtual Tissue Compiler**. The software displays the **Open** dialog box.
2. Navigate to the location of the AssemblyData.txt file.
3. Load this file. The software reads the instructions and creates the Virtual Tissue file, and displays a dialog box of its progress.

4. When complete, you have two files. The first file is the Virtual Tissue image in .jp2 format. The second files is an .xmp file, which contains metadata describing the file.

Displaying and Saving Virtual Tissues

Once a virtual slide has been acquired, it is automatically displayed in the Macro View window. Display virtual slides by clicking on the button. If the virtual slides are not displayed, right click in the Macro View window and select Display Acquired Images.

The image displayed in the Macro View window is automatically sized so that all of it fits in the window. This window can be resized and moved around the screen.

Position the cursor inside the Macro View window and click the right mouse button to bring up the **Go To** option. Click **Go To**, then click on the location in the Macro View window where you want to go, and you are taken to that field-of-view in the tracing window. If **Imaging>Live Image** is enabled, the

stage also moves to the new location. If you are viewing the virtual slide in the tracing window, the image is repositioned. A dashed white box shows your current location.

If **Move>Synchronize Stage and Images** is enabled, the stage also moved during any movements of the Virtual Tissue image, even if **Acquisition>Live Image** is not enabled.

Display virtual slides in the tracing window by selecting **Acquisition>Display Acquired Image** or clicking the button. Turn off display of the Virtual Tissue Image by returning to **Acquisition>Live Image**, or by deleting the image from the **Image Organizer.**

Saving Virtual Tissue Images

Stereo Investigator saves virtual tissue in

- MBF JPG200/JPG200 (*.jp2), all options
- TIFF (*.tif), 2D only
- Zoomify (*.pff) , 32-bit version of Stereo Investigator.

The Zoomify format allows Virtual Tissue size to be limited only by the size of the hard disk. Zoomify is not available in the 64-bit version of Stereo Investigator.

Tracing from Virtual Tissue

Tracing from virtual slides is similar to tracing from live images, except that you cannot change the focal depth unless you are using 3D Virtual Tissue. . Select **Acquisition>Display Acquired Image**, then use the Stereo Investigator tools as you would with a live image. Using **Move** commands or tracing outside the **AutoMove** area moves the image instead of the stage, keeping the tracing aligned with the image. Use the **Move Image** commands/buttons instead of **Joy Free** and **Joy Track** to move the image in the tracing window.

Zooming in and out of Virtual Tissue

When a virtual slide is displayed in the tracing window, the zoom buttons are enabled:

Zoom In: When this button is selected, a single click in the tracing window zooms in 2× around the point clicked. The point that is clicked moves to the center of the tracing window.

The cursor can also be used to drag a box that is enlarged to the size of the tracing window, allowing for variable zooming. Occasionally, the Zoom In appears to be less than expected. There are two reasons that the Zoom In is limited. A Zoom In includes all parts of the Zoom In box. When the shape of the tracing window and the zoom in box are drastically different expect the Zoom In to be limited. Windows does not display an image enlarged more than 20×, so to prevent this loss of image display, Stereo Investigator limits image enlargement to 19.5×.

Zoom Out: When this button is clicked, the image zooms out to 50% of its current size. The Zoom Out is done about the center of the tracing window.

The tracings associated with the virtual slide are still sized to fit the acquired image following a Zoom operation.

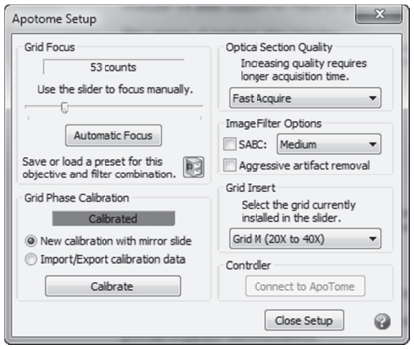
Chapter 16

The ApoTome Module

Before you start: Before using the Zeiss ApoTome, you should be familiar with its use. More information on the Zeiss ApoTome—including demonstration videos—is available at the Imaging and Components page at Carl Zeiss MicroImaging GmbH.

Setting up the ApoTome

Before you use the ApoTome, you need to set its operating parameters with the ApoTome Setup dialog box.



Which Settings Are Best to Use?

The settings you choose to use with the ApoTome depend on your tissue and its preparation, your study, and the time you have available. Only you can decide the settings best for your needs. Once you have correctly calibrated and focused the ApoTome there are a number of options for image collection that you can choose that best meets your needs. The general rule of thumb is that settings which increase the image quality also decrease the image acquisition speed. Therefore, chose the settings based on your specific requirements. For instance, to capture hundreds of image stacks for counting cells, it is more efficient to use a faster setting than you would selected for acquiring an image for a publication.

Grid Focus

Grid Focus is different for each objective and filter you use. Use the slider to manually focus the ApoTome in the light path until you find a position where the grid lines are sharpest in your live image. The software displays the number of counts used.
You can also use Automatic Focus.

To use Automatic Focus

- Click Automatic Focus. The software starts the automatic focus procedure and displays a number of counts.

Grid Focus Presets

You can save presets , edit them, and load previously saved presets. You'll need to save presets if you do multi-channel acquires, since the Device Control Sequence dialog requires these presets to run.

We highly recommend finding and saving the best grid focus for each objective and fluorescent filter combination that you will use as part of the initial setup. Loading presets for particular combinations is faster and easier than searching for the best focus each time you change the filter or objective. For multichannel acquires, this is a requirement so that the software knows where to position the grid for each channel.

To save or delete presets

1. Click the **Presets** button and choose **Edit Presets**. The software displays the Preset Save/Update dialog box.

2. Type a name for the preset, and then click **Save** or select an existing preset and click **Delete** to remove it.

You can use an existing preset to jump to a previously saved focus position.

To load an existing preset

- Click the Presets button and choose a preset from the list.

Optical Section Quality

If you want increasing quality, you need more input images. Increasing the image quality can reduce grid line artifacts as well as reduce noise similar to averaging images, at the expense of longer acquisition times. You can choose from these quality values:

- Fast Acquire—3 input images acquired to create the single, final image

- Good Quality—5 input images acquired to create the single, final image

- Better Quality—7 input images acquired to create the single, final image

- Best Quality—9 input images acquired to create the single, final image

Image Filter Options

Acquired images may have artifacts burned (or "bleached") into the tissue from the acquisition process. To clean up bleached in grid lines, you can use the SABC (Spatially Adaptive Bleaching Correction) filter. The SABC options (Weak, Medium, Strong) progressively remove more artifacts.

Aggressive artifact removal removes the most stubborn artifacts. Use it carefully—it may remove some of your sample from the image.

Grid Insert

If you change the grid currently installed in the ApoTome slider, change this setting. You do not need to recalibrate.

Grid Phase Calibration

If you need to recalibrate, use the calibration slide and filter, choose New calibration with mirror slide.

You can also import and export calibration information. We recommend backing up good calibrations to avoid having to repeat the process in the event your system configuration is lost or corrupted. Choose **Import/Export calibration data** and click **Import** or **Export**.

Controller

Click **Connect** to connect to the ApoTome.

Close Setup

Click **Close Setup** when you are finished changing the setup options.

Chapter 17

Stereological Probes: Foundations

Stereological methods are precise tools for obtaining quantitative information about three-dimensional, microscopic structures, based mainly on observations made on sections. (H.J.G.Gundersen et. al, 1988)

Two important concepts in stereology are "unbiased" and "efficient". According to Gundersen et. al., *The concepts are used in a statistical sense and mean 'without systematic deviation from the true value' and 'with a low variability after spending a moderate amount of time', respectively.*

Stereo Investigator is used to execute unbiased, efficient*, Design-Based Stereology* protocols in order to estimate areas, volumes and population counts, etc.

You may find it useful to follow these steps:

- **Determine exactly what property you want to estimate and determine the best probe to use for the quantity you want to estimate.** For instance, if you want to count cells, you should determine if you want an estimate of absolute cell population, or an estimate of cell density. Read Estimating and Measuring with Stereo Investigator for assistance.
- **Familiarize yourself with what your probe requires.** Read Background Concepts for Using Stereological Probes to familiarize yourself with the components and procedures that are required for the probes that you have chosen to use. The concepts of systematic random sampling, region of interest (ROI), counting frame, guard zone, and others are explained in this section.
- **Learn about proper tissue preparation early in the planning process.** Many probes have specific requirements for tissue preparation; read Tissue Preparation for Stereological Probes for background and other information.
- **Learn about using Coefficient of Error to determine accuracy.** Using Coefficient of Error explains the different CE calculations and how to interpret them.

Estimating And Measuring With Stereo Investigator

This section describes many of the various methods Stereo Investigator uses to perform measurements and estimates. A list of the quantities that can be estimated using Stereo Investigator is at the end of this section.

Area

Stereo Investigator allows you to trace a closed contour around a region of interest. It will then calculate the area, as well as numerous other morphometric properties of this region.

For those wanting to use a stereological protocol, there are global probes, such as the Cavalieri estimator, that can be used to estimate the area of a region of interest. Additionally, there are local probes, such as the Nucleator, that can be used to measure the area of particles.

Volume

In order to estimate the volume of a region of interest in a single section, Stereo Investigator can be used to measure the section thickness. This (average) thickness can then be used with the Cavalieri estimator to estimate volumes.

This thickness value can also be used in conjunction with the area measurements of contours traced around regions of interest on one or more slides to estimate the volume of a region of interest.

Local Probes

Volume estimates of smaller objects, such as cells, may also be made using local probes. These local probes include the Nucleator, Planar Rotator, and the Optical Rotator.

Population Counts

There are several stereological probes that can be used to estimate the population of objects across an area or a volume. These probes include the Optical Fractionator and the Physical Fractionator (formerly the Physical Disector). The thickness of tissue sections is a key determination of which probe to use. The optical disector is for use on thick sections, while the physical disector is used on thin sections.

Population Estimates within an Area

If you want to estimate a population that lies within a 2D region of interest, you can do so by several means.

Population Estimates

If the area of the region of interest is not known, or cannot be determined accurately, a valid population estimate can be achieved using the Optical Fractionator protocol.

Population Density Estimates

If you want to determine aerial population density within a 2D region of interest whose area is known (or can be accurately computed), the Optical Fractionator protocol can again be used. However, the resulting statistics are used with formulas that require the area of the region of interest, the sampled area and the number of sampled objects, etc.

Population Estimates within a Volume

As with area based estimates, there are several different protocols, and different formulas can be used with each protocol.

Population Estimate

A major advantage of the Optical Fractionator is that it can be used to estimate total population count without having to accurately measure the volume that is being sampled.

Volume Density Population Estimate

If you want to determine population density within a region of interest whose volume is known (or can be accurately computed), a slightly modified version of the Optical Fractionator protocol can be used. This method is often referred to as the N_v:V_{ref} technique. The resulting statistics are used with formulas that require the volume of the region of interest, the sampled volume and the number of sampled objects, etc. The volume of the region of interest is obtained by tracing it with a contour in Stereo Investigator. The reference volume is calculated using the planimetry method, which calculates the area within the contour automatically and uses the Cavalieri method to estimate volume.

What Kids Of Object Properties Can Stereo Investigator Estimate?

The following is a list of object properties that can be estimated using the probes in Stereo Investigator.

For…	Use…
Object Number	Physical Fractionator, Fractionator, Optical Fractionator, Schmitz Nearest Neighbor
Length	L-Cycloid Optical Fractionator, IUR Planes Optical Fractionator, Space Balls, Isotropic Virtual Planes
Length/Volume	Cycloids for Lv, Isotropic Virtual Planes
Profile Area	Optical Fractionator with contour trace, Cavalieri Estimator, Combined Slope Intercept, Nucleator, Area Fraction Fractionator
Surface Area	Isotropic Fakir Method, Surfactor, Optical Rotator
Surface Area/Volume	Cycloids for Sv, Merz (half-circle test system), Weibel (dash test system)
Area Fraction	Cavalieri, Area Fraction Fractionator
Volume of Tissue	Optical Fractionator with contour trace, Cavalieri, Surface Weighted Star Volume, Vertical Spatial Grid, Nucleator
Volume of Cells	Point Slope Intercept, Discrete Vertical Rotator, Nucleator, Planar Rotator, Optical Rotator
Volume Fraction	Cavalieri, Area Fraction Fractionator
Spatial Distribution	Area Fraction Fractionator

Background Concepts For Using Stereological Probes

Before using any of the probes in Stereo Investigator, you should familiarize yourself with these background concepts.

Regions Of Interest

All stereological probes in Stereo Investigator that use systematic random scans throughout regions that are larger than one field of view require that a region of interest be identified. This procedure involves defining the boundary of the region. An area is circumscribed by a closed contour surrounding the region of interest. Contour tracing is easily done with the Stereo Investigator.

Accuracy Of Area And Volume Estimates

Global stereological probes can be used to estimate areas and volumes. In addition to performing area and volume estimates, some of these probes also provide an estimate of the precision of their estimations.

Refer to the section on the Cavalieri estimator for more information on area and volume estimation using stereological techniques.

The area estimation of a closed contour mapped with Stereo Investigator is more precise than that given by the Cavalieri estimator.

A volume is delineated by its bounding surface that, in Stereo Investigator, is defined by a series of closed contours in different tissue sections.

Volumes and surfaces are usually visualized in microscopy as a sequence of serial sections (optical or physical). Stereo Investigator uses the conventional technique of representing a volume as a sequence of slices, each being defined by the contour traced at its upper or lower surface and by the thickness of the section. It is thus easy to define a volume of interest using Stereo Investigator by tracing a region of interest appearing in several sections.

How Do I map A Region Of Interest?

Mapping a single region by tracing a contour around it is a simple task.

Make sure that the objective lens you are using is selected in the lens selection pane on the Main toolbar.

If you are using a video camera, make sure that the live video image of your slide appears on the computer screen. If not, click **Imaging>Live Image**.

1. Select a contour name from the contour list on the **Main** toolbar.
2. Click to select a reference point.
3. Move the mouse cursor and left-click on any location along the edge of the region.
4. Move the mouse and click on the successive points along the perimeter of the region of interest. If you are using continuous tracing mode, you may keep the left button pressed while moving the mouse.
5. If the region of interest extends beyond the field of view, you can click **Move>AutoMove**, so that Stereo Investigator moves the stage and the tracing simultaneously as you trace towards the edge of the screen. This automatically moves more of the region of interest into the current field of view.
6. Once you have finished tracing the contour, click the **Close Contour** button on the **Main** toolbar, or right-click **Close Contour**. This automatically connects the last point traced to the first point traced. The area and perimeter of the contour is automatically calculated. Click the **Contour Measurements** button to view the results in the **Contour Measurements** window. While tracing a contour, the distance separating sequential points along the perimeter is up to you. It can be variable, with relatively large distances separating points along reasonably straight segments and short distances separating points at highly curved locations. Point separation also can be constant, or as nearly so as you can manage by eye. Constant point separation may be maintained by setting the circular cursor to a specified radius. Then tracing a new point only when the outer edge of the circular cursor lies over the previously traced point.

What Is Systematic Random Sampling (SRS)?

The concept of systematic random sampling lies at the heart of many stereological protocols.

A systematic random sampling procedure provides an unbiased and efficient sampling technique. It is better than other forms of sampling when multiple sampling runs are implemented, either for a given region of interest, or over several regions of interest as is done when multiple tissue groups are examined.

Once the shape and size of a sampling area or volume is chosen and a region of interest has been determined, sampling sites are evenly distributed throughout the region of interest. The distribution pattern of the sampling sites is systematic, i.e. the distance from each sampling site to the next is the same. The placement of this pattern of sampling sites is a random component.

Using this technique, if a region of interest is sampled more than once, or if a series of similar regions of interest are sampled, the sample area/volume at each sample site will remain the same and the distance between the sample sites will remain the same. But the placement of the systematic sampling scheme for each sample run will be randomized.

Systematic Random Sampling of Serial Sections

Systematic random sampling of sections means that you set up a random selection process to pick out a small number of uniformly spaced sections from a complete sequence of sections. For instance, if you have 30 sections you might choose to sample every 4th section (i.e., a section increment of 4). The first section chosen would be a random selection of 1, 2, 3, or 4. The choice of the section increment (and the exact number of sections to sample) is dictated by considerations of estimation precision.

With this procedure, the section increment is the systematic element and the choice of which section to start with is the random element.

When several series of sections are to be sampled, it is important to randomly select a new starting section for each series, while using the same section increment for all series. For instance, if your random starting index for the first series is 3, you would start with the 3rd section of the first series and then sample every 4th section. You would then want to pick a new random section between 1 and 4 as the starting section for the second series and then sample every 4th section. Therefore, the first series would use sections 3,7,11, etc. and the next series might use sections 1,5,9, etc.

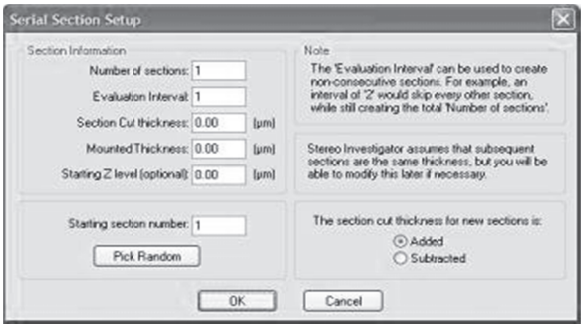
How to set up a systematically random sample of serial sections

For this example we assume that the total number of sections, the section increment and the thickness of each section is known.

1. Start Stereo Investigator, select a lens, and mark a reference point.
2. Click **Tools>Serial Section Manager**.
3. Click the **New Section** button.

   The **Serial Section Setup** dialog box prompts you to enter the necessary information:

   - The section thickness.
   - The section interval that you want to sample.
   - Select the sequence number of the first section. You can either choose a section yourself or click the Pick Random Starting Section button to determine one for you.
   - A Section Name. A default name is automatically created. You may use this name or replace it with your own more meaningful name.



5. Click **OK** and then click the **Close** button.
6. Once you have used the Serial Section Manager to define the initial serial section sequence information, you can use it again at a later time to specify each new section that you analyze.

Important Note! — When you are sampling several sections in a single specimen, it is essential to use the same sampling parameters for all sections. This means that for all sections of a given specimen, the grid size (XY Spacing of Sampling Sites) and counting frame size must be the same for a given region of interest. When sampling multiple specimens, the sampling parameters do not need to be the same between different specimens, just within any given specimen.

To specify each new section

1. Click **Tools>Serial Section Manager**.
2. Click the **New Section** button. Stereo Investigator automatically updates the section number, section depth and section name values. You should verify that these values are correct or override these values as necessary.
3. Prepare to view the new section that is specified in the dialog box. If you would like to see only the tracing of the new section that you will be working on, select the **Only Display Current Section** option in the **Serial Section Manager** dialog box before clicking **OK**.
4. Click **OK**.

Across Serial Sections

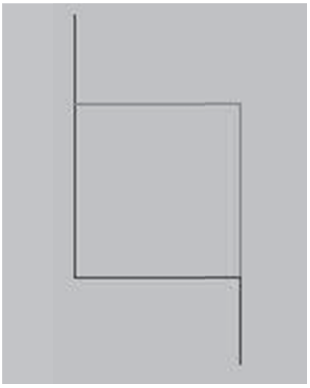
When sampling areas of interest across more than one serial section you will first determine which serial sections to sample, as discussed above (this is a systematic random selection of sections). Then execute the selected protocol for each region of interest in each section (this involves a systematic random selection of sampling sites).

What Are Counting Frames?

Many stereological protocols utilize either a 2D counting frame or a 3D counting frame. The former is a 2D entity, while the latter adds the Z-axis (focal depth) to become 3D.

The 2D Counting Frame

A counting frame is a graphical projection used at each sampling site with the Disector, Optical Disector, Physical Fractionator, Optical Fractionator, and other probes as an aid in unbiased counting of objects for population estimates. It helps ensure an unbiased estimate by preventing over counting. As used with Stereo Investigator, it is a rectangular region with two tails.



The tails are an upward extension of the left side of the counting frame and a downward extension of its right side. The left and bottom sides of this rectangle and the tails are considered forbidden lines and objects that touch these sides or either of the tails should not be counted. Objects that lie within the counting frame and/or cross the top and right sides without crossing the forbidden lines can be counted.

The 3D Counting Frame

A 3D counting frame is a counting frame that is projected along the Z-axis. It is a 3D entity whose thickness should never exceed the actual physical thickness of the section to which it is applied. It is used by the protocols that employ the Optical Disector, such as the Optical Fractionator and the Linear Optical Disector.

You will need to determine the specific dimensions of the counting frame before using these probes.

Guard Zones

When using an optical disector, the counting frames are usually positioned so that their upper surface (along the Z-axis) is a specific distance below the top surface of the section, and their lower surface is above the bottom surface of the section. The region that lies between the top surface of the section and the upper surface of the counting frame, and the region that lies between the bottom surface of the section and the lower surface of the counting frame are known as guard zones.

The size of the guard zones can depend on many factors. Their primary purpose is to eliminate areas that are not representative of the tissue specimen from the sample volume. For instance the microtome commonly causes many types of surface aberration: tearing, lost caps, etc. Guard zones should be large enough to exclude any volume at the top or bottom of the section that may have been affected in this way.

Remember that shrinkage along the Z-axis may be extreme relative to that of the X and Y-axes.

For a detailed discussion see: Helander K.G., Thickness variations within individual paraffin and glycol methacrylate sections (1983) J. Microscopy, Vol 132:223-227.

Overlays

Overlays of various geometric shapes are used by all stereological protocols as an aid to performing estimations.

Detailed explanations of each of these overlays are given with the descriptions of the probes that use them since each overlay is unique to a given probe.

Tissue Preparation For Stereological Probes

Many probes have specific requirements for tissue preparation. These include sectioning orientation, thickness and selection of sections. Be sure to review the tissue requirements as early in your experimental procedure as possible. Many users have been disappointed to find they have sectioned inappropriately for the probe they want to use. The need to repeat experiments can be avoided with careful planning.

Section Thickness

The Optical Fractionator is the most commonly used stereological probe in Stereo Investigator. As it uses optical fractionating of the tissue, the tissue must be thick enough to provide multiple optical sections. Generally, tissue should be at least 20 microns thick after processing and mounting. Don't underestimate tissue shrinkage in the Z-axis; it is not uncommon for tissue to shrink down to 25% of its original thickness during fixation and dehydration. Sections as thin as 10 microns can be used with the Optical Fractionator, but this involves significantly shrinking the size of the guard zones, which may or may not be acceptable in your tissue.

Other probes requiring thick sections are:

- All Optical Fractionator probes
- Cycloids for Lv
- Space Balls
- Isotropic Virtual Planes
- Isotropic Fakir Method
- Vertical Spatial Grid
- Surfactor
- Optical Rotator
- Schmitz Nearest Neighbor

Section Orientation

Random orientation of particles to be measured or counted is a requirement for several stereological probes. For this to be achieved, the particles must either be in true random orientation (an assumption you would need to support in your results), or the tissue must be sectioned at a random orientation. Two kinds of random sectioning are commonly employed in stereological protocols: Isotropic Sectioning and Vertical Sectioning.

Isotropic Uniform Random (IUR) Sectioning

Isotropic sectioning is random in X, Y and Z. This can be accomplished by randomly orienting the tissue prior to sectioning and then sectioning at a regular interval. Uniform random sectioning (also called systematic random sampling) means that the start point is randomly determined, but that subsequent sections are at a uniform distance from each other.

Vertical Uniform Random (VUR) Sectioning

Sometimes the nature of the tissue or the nature of the particles to be measured makes IUR sectioning impractical (for example, if it becomes impossible to identify the region of interest). VUR sections are generated by setting a vertical axis, then rotating the tissue around the vertical axis in a random fashion before sectioning. Sections are then uniform random (uniform distance with a random starting point). Methods using VUR sections require identification of the vertical axis within each tissue section.

Preferential Sections

Some probes can be used with preferential sections, that is, sections that are in a specific orientation, such as horizontal or coronal sections. Some probes work with sections of any orientation because the assumption of isotropy, uniform properties in all directions, is not necessary in their calculations. These probes include:

- Cavalieri
- Physical Fractionator
- Fractionator
- Optical Fractionator

Computer-assisted microscopy has enabled the use of preferential sections for analyses previously only accessible to IUR sections by generating IUR virtual planes through thick tissue sections. The orientation of IUR sections is replicated by changing the orientation of the probe rather than the orientation of the tissue. Probes that work in this way include:

- Space Balls (an isotropic probe working in tissue of any orientation)
- Isotropic Virtual Planes
- Isotropic Fakir Method

Using Coefficient Of Error

The coefficient of error (CE) is a method for estimating the final accuracy of a probe run. The coefficient of error takes into account the distribution of particles in the tissue, the inter- and intra-section variation, and the total number of particles counted or sampled. There are several coefficients of error commonly used in Stereology. The Gundersen CE is the most commonly used, but we recommend following the CE protocol established within your field, and reading the CE references (at the end of this section) to determine which CE best fits your needs.

CE Gundersen

This CE is only displayed if three or more sections are sampled. There are two values for many of the Gundersen categories, m=0 was used in the original CE calculations (Gundersen,1987), m=1 is used in the newer CE estimate formula (Gundersen, 1999).

- **Variance Due to Noise:** Formerly called the Nugget Variance, also known as the Nugget Effect, this is a measure of the intra-sectional variance. It is a primary contributor to the Total Variance. Ideally, this value will be both small and of roughly the same magnitude as the Variance Of

Systematic Random Sampling. If this value is too high, more sampling needs to be performed on each section.

- **Variance of Systematic Random Sampling:** This is a measure of the inter-sectional variance. It is a primary contributor to the **Total Variance**. Ideally, this value will be both small and of roughly the same magnitude as the **Variance Due to Noise**. If this value is too high, more sections will need to be sampled. At least three sections will need to be sampled for this value to be statistically meaningful. If less than three sections are displayed, these values are not shown in the results. Although this value depends to some degree upon the **Variance Due to Noise**, this dependency will normally be very small if the **Variance Due to Noise** is reasonably small.
- **Total Variance:** This is the sum of the **Variance Due to Noise** and the **Variance Of Systematic Random Sampling** over all the sections sampled. This value is the primary contributor to the final **Coefficient Of Error** for a given estimate over a series of sections. At least three sections will need to be sampled for this value to be statistically meaningful.
- **Coefficient of Error (Gundersen):** This value represents the estimated precision of the population size estimated using the optical fractionator. At least three sections must be sampled for this value to be computed.

CE Scheaffer

- **Estimated Mean Cell Count:** This is the mean number of cells per counting frame across one or more sections.
- **Estimated Variance of Cell Counts:** This represents the estimated variance of the number of cells per counting frame over one or more sections. This is an indicator of how evenly the cell count was distributed over the counting frames.
- **Estimated Variance of Mean Cell Counts:** This represents the estimated variance of the estimated mean number of cells per counting frame from the true mean. A sufficient number of counting frames should be used to ensure that this number is small.
- **Contour Area / Counting Frame Area:** This ratio indicates an inverse of the fraction of the total area actually sampled.
- **Estimated Cell Population Count:** This value for the estimated population size is computed by multiplying **Contour Area / Counting Frame Area** by the **Estimated Mean Cell Count**. This is a simple linear, biased, approximation.
- **Estimated Variance of Estimated Cell Population:** This is the variance of the population size estimated using the linear approximation formula for **Estimated Cell Population Count**.
- **Estimated Coefficient of Error (Scheaffer):** This value, based on equations published by R.L. Scheaffer, provides an alternative method of estimating the coefficient of error for random populations. The calculations for this estimated CE are not unbiased if the population distribution is non-random, or if the contours circumscribing the regions of interest are not accurately traced.

CE Cruz-Orive

- **Estimated CE (Cruz-Orive/Geiser):** This value, based on equations published by M. Geiser and L.M. Cruz-Orive, provides an alternative method of estimating the coefficient of error.

CE Schmitz-Hof

These values, based on equations published by C. Schmitz and P. Hof, provide an alternative method of estimating the coefficient of error.

Other Results

- **Z Depth Histogram:** Shows the Z depth location for place markers in each section.
- **Planimetry:** These values show how area and volume are calculated based on the area contained within the traced contour. Variance Details are also provided.

References

Links in the reference list go to PubMed AbstractPlus entries.

- Schmitz C, Grolms N, Hof PR, Boehringer R, Glaser J, Korr H. (2002). Altered spatial arrangement of layer V pyramidal cells in the mouse brain following prenatal low-dose X-irradiation. A stereological study using a novel three-dimensional analysis method to estimate the nearest neighbor distance distributions of cells in thick sections. Cereb Cortex. 2002 Sep;12(9):954-60.
- Geiser, M., L.M.Cruz-Orive, and I.M. Hof (1990) Assessment of particle retention and clearance in the intrapulmonary conducting airways of hamster lungs with the fractionator. Journal of Microscopy 160 (Pt 1): 75-88.
- Glaser, E.M. and P.D. Wilson (1998) The coefficient of error of optical fractionator population size estimates: a computer simulation comparing three probes. Journal of Microscopy. 192(Pt 2): 163-171.
- Gundersen, H.J.G. and E.B.V. Jensen (1999) The efficiency of systematic sampling - reconsidered. Journal of Microscopy. 193(Pt3): 199-211.
- Gundersen, H.J.G. and E.B.V. Jensen (1987) The efficiency of systematic sampling in stereology and its prediction. Journal of Microscopy. 147(Pt3): 229-263.
- Schmitz, C. and P. Hof (2001) Recommendations for straightforward and rigourous methods of counting neurons based on a computer simulation approach. Journal of Clinical Neuroanatomy. 20:93-114.
- West, M.J., L. Slomianka, and H.J.G. Gundersen (1991) Unbiased stereological estimation of the total number of neurons in the subdivisions of the rat hippocampus using the Optical Fractionator. The Anatomical Record 231: 482-497.

The Disector And Optical Disector

The Disector

The disector is a 3D stereological probe that has one unique and very important attribute, it samples objects with a probability that is proportional to their number, not their size. Because of this, the disector can be used to count objects without having to make any assumptions about their size, shape, or orientation. In its simplest form, the disector involves the use of two sections, hence the name disector.

The counting rule used with the disector probe is simple. One counts the objects that are present on the second section of a pair of adjacent sections that are not present on the first, as one proceeds systematically through a series of sections that includes the region under consideration. An equally valid counting rule is to count objects that are observable in the first section and not in the second. Essentially what one is doing is determining whether or not a unique point on each object, the "top", or as in the second case, the "trailing edge", lies within the volume sampled by the disector.

In Stereo Investigator the disector is not a stand-alone probe, but is used as an integral part of other probes, such as the physical disector and the linear disector. Designing a disector involves defining the counting frame size.

Defining the Counting Frame

When a probe that uses the disector is invoked, Stereo Investigator automatically positions the counting frame for each sampling site within the region of interest.

To define a counting frame

1. Click **Probes>Define Counting Frame**.
2. Choose to either **Define Graphically** by dragging the edges of the counting frame to the desired size, **or Define Numerically** by specifying the XY dimensions of the counting frame in the dialog box.
3. If you would like to use a square counting frame, select **Force counting frame to be square** option on the **Counting Frame** tab in the **Options>Stereology Preferences** dialog box. This causes the counting frame to appear as a square when defining it graphically, and presents only a single dimension when defining the counting frame numerically.

Reference

- Sterio, D.C. (1984) The unbiased estimation of number and size of arbitrary particles using the disector. J. Microsc. 134: 127-136

The Optical Disector

The concept of the optical disector is an extension of the original disector. Instead of observing two physical sections as in the disector, one observes optical sections. It is possible to optically section thick histological sections by using microscope objectives with high numerical apertures that produce images with relatively narrow depths of focus. The focal plane (or optical section) can be moved a known distance through the thickness of the section, producing in effect, a continuous series of superimposed sections within which counting could be carried out with disector counting rules.

In practice this consists of counting the number of new objects that come into focus (or alternatively, disappear) as one focuses through a known volume of the tissue. The motion through the focal plane axis is determined using a stepper motor or a position encoder mounted on the microscope.

The optical disector has one very important advantage over the physical disector: it eliminates the difficult and time consuming task of identifying corresponding parts of two physical sections (i.e., determining whether particular object can be seen on one section and not the other). With optical disectors, the sections are always optimally positioned for comparisons. This means that it is easier to fulfill the first requirement for the application of the disector, i.e., the ability to identify the sectional profiles of the same

object on the sections used in the analysis. One simply focuses up and down, prior to counting, to establish the relationship between profiles at different levels. This capability is extremely valuable, if not essential, when branching or highly irregular objects are being counted. With physical disectors, this process may require the preparation of additional sections between those used for counting.

The optical disector is primarily used in conjunction with other probes. For instance, the optical fractionator is a combination of the fractionator and the optical disector. Likewise, the linear optical disector is a combination of the linear scan with the optical disector.

Although it is not used independently in Stereo Investigator, the optical disector is an important stereological probe in its own right and is described here as such.

Unbiased sampling of a Volume

The optical disector is a technique for counting particles in a small sample volume. It uses a 3D counting frame and a counting rule that defines when particles inside the counting frame are to be counted and when they are to be ignored.

Guard Zones

The counting frame must enclose a representative volume. Due to irregularities that occur at the surface of a tissue section (lost caps, warping, tearing, etc.), the top plane of the counting frame should be located below the top surface of the section. The region between the top of the section and the top of the counting frame is called the guard zone.

Normally, it is desirable to have a guard zone both above and below the counting frame.

Counting Rules

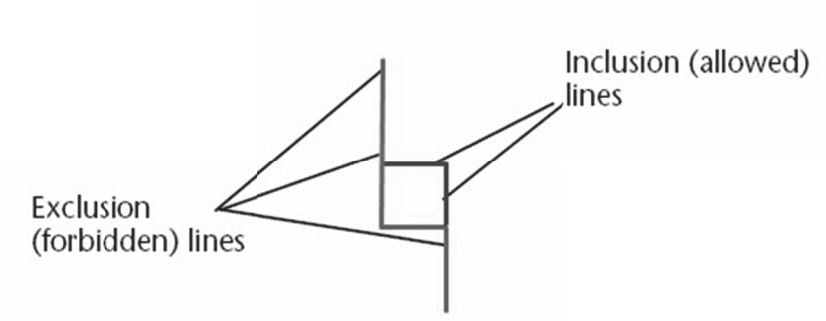
Observing the counting rule ensures unbiased counting. If a series of optical disectors were situated next to each other, no particle would be counted more than once, even if part of a particle existed in more than one counting frame.

The first counting rule is to only count particles that come into focus as you focus down from the top of the counting frame (assuming you are scanning down through the counting frame). Particles that are in focus above and at the top of the counting frame should not be counted. If a particle comes into focus inside the counting frame, but extends down past its bottom face it can be counted.

The second counting rule defines three neighboring faces of the counting frame's six as forbidden. A particle is ignored if any part of its surface crosses these faces. Particles that cross any of the other three faces are counted. Conventionally, the counting frame's forbidden faces are the left YZ face, the lower XZ face, and the top XY face. In addition, extensions of the left and right YZ faces (z projections of the counting frame tails) exclude the counting of particles that cross the permitted faces and then curve around to cross these extensions. The forbidden faces and extensions extend down past the bottom of the counting frame by a distance equal to the height of the counting frame.

An alternative to the forbidden line rule is the unique point rule; the unique point rule requires that a unique and arbitrary point can be associated with each particle being counted. For instance, a unique point i.e., the top/left-most portion of the particle, or its nucleolus (if the particle is a cell and every cell has one point-like nucleolus) is used to determine if the particle is inside the counting frame. In this case it is still necessary to utilize the forbidden faces of the counting frame, but the extensions are not needed.

The following illustrations may help explain the concept of the forbidden and allowed surfaces of the counting frame.



A typical counting frame. This illustration shows a typical counting frame for sampling particles. Any particles that fall entirely inside the square are to be counted, but particles touching or crossing the boundaries must follow the counting rules. The top and right surfaces of the square are the inclusion (allowed) lines, the left and bottom surfaces are the exclusion (forbidden) lines. In addition, the exclusion lines continue to infinity up and down from the right and left sides of the square.

The counting rules for this type of counting frame are as follows:

All particles falling entirely within the square are to be counted, or particles with their "unique point" falling entirely within the square.

Decide whether you will use touching or crossing when counting or not counting particles. Choose one, but not both. Be consistent in counting/excluding. For example, If you count particles touching the inclusion line, you must not count particles counting the exclusion line, even if it does not cross the line.

The following illustration shows an array of particles in and around a counting frame:

Notice the position of the particles in relation to the counting frame elements.
Particles with a check mark are to be counted—they touch the inclusion lines.
Particles with a x are not to be counted—they either fall entirely outside the counting frame, or touch the exclusion lines.
If a particle touches both the inclusion and exclusion lines, it is not to be counted.

Visualizing Counting Frame Rules

One way that is helpful in remembering the counting frame rules is to remember that in stereological sampling, each particle or cell must have one and only one counting frame in which it can be counted, and it cannot be counted in any other location. Your counting frame has fallen in a particular location, but imagine that there is a grid of adjacent counting frames all around it. If the particle in question could be counted in any adjacent frame, it cannot be counted in the current frame. For example, the crescent shaped particle in the above illustration does cross the inclusion line, so why not count it? Imagine another counting frame directly beneath the one illustrated. In this counting frame, the crescent particle would cross the inclusion line and be counted. Therefore, it should not be counted in the current frame. If a particle could be counted anywhere else, do not count it.

Designing An Optical Disector Probe

Designing an optical disector involves defining the counting frame size (this is the counting frame in the XY plane and then a thickness), and then specifying height of the upper and lower guard zones.

Defining the Counting Frame

When a scanning probe that uses the optical disector is invoked, Stereo Investigator automatically positions the counting frame for each sampling site within the region of interest.

1. Choose **Probes>Define Counting Frame.**
2. Type the counting frame's X and Y dimensions.
3. Choose either **Define Graphically** or **Define Numerically** to specify the XY dimensions of the counting frame.
   Define Graphically lets you specify the size of the counting frame with the mouse. Its dimensions are determined from the screen coordinates of two mouse clicks you make (at the upper left and lower right hand corners of the desired counting frame). Stereo Investigator automatically adds the extension lines. After placing the upper left corner of a counting frame, holding down the SHIFT key while moving the mouse allows you to change the position of the counting frame.
4. If you want to use a square counting frame, select **Force counting frame to be square** option in the **Counting Frame** tab of the **Options>Stereology Preferences** dialog box. Alternatively, right-click while you are defining the counting frame and select that option from the context sensitive menu. This causes the counting frame to appear as a square when defining it graphically, and presents only a single dimension when defining the counting frame numerically.
5. Click **OK**.

Guard Zone and Thickness

The thickness of the guard zones and the counting frame thickness are constrained by the actual section thickness and an evaluation of how much shrinkage the section has experienced along the Z-axis after processing.

You are prompted to enter the section thickness, the counting frame thickness and the top guard zone thickness when you preview or run a probe that utilizes the optical disector.

Reference

- West, M.J., L. Slomianka, and H.J.G. Gundersen (1991) Unbiased stereological estimation of the total number of neurons in the subdivisions of the rat hippocampus using the Optical Fractionator. The Anatomical Record 231: 482-497.

Chapter 18

Stereological Probes

Stereo Investigator Workflows

Stereo Investigator includes Workflows, automated helpers to assist you with your work. Each Workflow leads you through using a specific probe. It directs your steps, and if you enter any information that does not "make sense" to the Workflow, it lets you know where you have gone wrong and how to fix it.

We designed workflows to stay out of your way while you work. Some of the probes or acquire procedures can involve many separate commands and decisions. Instead of memorizing a sequence of commands or having to do work before using a probe, workflows contain all the commands and asks only for the information necessary to complete a task.

Many of the workflows share steps. The more you work with the workflows, the easier it becomes to use them.

For complete help with the workflows, please see the online Help that accompanies each workflow.

Define Counting Frame

This allows you to define the XY size of the counting frame that is used with the Fractionator, Optical Fractionator and Linear Optical Disector probes. You can do this in two ways:

- **Numerically**, by entering specific values for the X and Y dimensions of the counting frame.
- **Graphically**, by changing the size of the counting frame in the trace window using the mouse to drag the edges or corners to the desired locations.

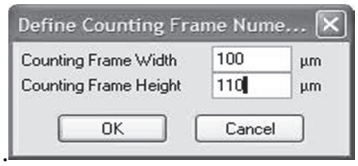
The red lines are the forbidden boundary. Objects that cross them are not counted. The green lines form the permitted boundary. Particles that cross them can be counted if they do not also cross a forbidden boundary.

If the counting frame is not visible in your tracing window, it may be larger than the current field of view. Use the **Define Numerically** procedure outlined in the next section to change the Width and Height values to smaller numbers until the counting frame comes into view.

The counting frame disappears immediately after it has been defined. It is only displayed once during the execution of the probes that use it.

Define Numerically

One method of defining the counting frame's dimensions is by specifying the size in the **Define Counting Frame Numerically** dialog box



If the **Force counting frame to be square** option in the **Counting Frame** tab of the **Stereology Preferences** dialog box is enabled, you can only enter one numeric value—the size of the counting frame square.

Define Graphically

You may specify the size of the counting frame using the mouse cursor and the left mouse button to move the borders of the frame in the XY plane. When the cursor changes to a double headed arrow, left-click to move the edges in or out. When the cursor changes to a diagonal double headed arrow, left-click to move the corners (2 sides will move together). When the cursor changes to a four headed arrow, left-click to drag counting frame to a new location within the tracing window. Any changes to the size of the counting frame are automatically updated in the Define Counting Frame Numerically dialog box.

There is an alternate method for defining the counting frame that is a variation of the method used in previous versions of Stereo Investigator: Click **Probes>Define Counting Frame**. Left-click anywhere outside of the currently displayed counting frame to set the upper left boundary of the counting frame, and then click a second time to define the lower right boundary. When defining the counting frame with this method, Stereo Investigator displays the counting frame area in the center panel of the Status Bar. You can then adjust the parameters of the counting frame as described previously.

Right-button options

- **Change Left Colors:** This is normally red, indicating the forbidden boundary. You can change this color if it is too difficult to see clearly.
- **Change Right Colors:** This is normally green, indicating the permitted boundary. You can change this color if it is too difficult to see clearly.

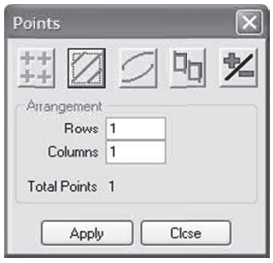
The color settings can also be changed on the **Options>Stereology Preferences Counting Frame** tab.

- **Force Square:** The counting frame will be constrained to be square, i.e. the horizontal and vertical dimensions will be equal, if this option is chosen.
- **Accept Count Frame:** Accepts the current size and location of the counting frame.
- **Cancel:** The definition of a new counting frame can be canceled using this option.

Define Made to Order Layouts

Define Made to Order Layouts allows you to design your own probe layout out of a toolbox that includes counting frames, cycloids, points, and lines. In addition, you can save layouts for later use by using the **Presets** option.

When you choose **Probes>Define Made to Order Layouts**, Stereo Investigator displays the **Made to Order** dialog box.



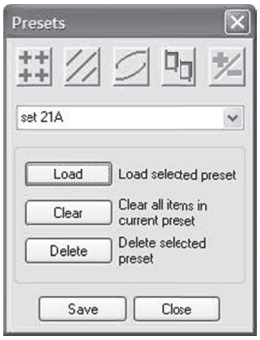
Each of the buttons at the top of the dialog box allows for the selection of different modalities: Points, Lines, Cycloids and Counting Frames, as described below.

Made to Order Layout Types

- **Points:** To use a grid of points in your probe, select the arrangement of points to use and click **Apply**. If you want a grid of points that are a set distance apart, the Cavalieri Probe is available.
- **Lines:** Set the number of lines and the angle to horizontal (for example, setting the angle to 90 generates vertical lines).
- **Cycloids:** Set the size of the cycloid as a percentage of field of view, and determine the orientation.
- **Counting Frame:** The counting frame is set as a percentage of the field of view.

Saving Made to Order Presets

A **Made to Order Layout** must be saved as a preset before the **Made to Order** probe is available on the **Probes** menu. The options available in the **Presets** dialog box:



- **Save:** Once all of the graphical components of a **Made to Order** probe have been chosen, type in a name for the layout and click **Save**. Stereo Investigator saves the **Made to Order** probe, which is now available for all files opened by Stereo Investigator.
- **Load:** Once **Made to Order** layouts have been saved, they can be loaded for inspection or editing. Select a layout from the drop-down menu in the **Presets** dialog box and click **Load**. Stereo Investigator displays the layout, and changes can be made. The probe cannot be run from this dialog box; to run the probe, choose **Probes>Made to Order**, click the **Presets** button, and select the desired layout.
- **Clear:** Click this button to clear all displayed elements. The preset file is not deleted.
- **Delete:** Deletes the **Made to Order** layout selected from the drop-down menu.

Preliminary Population Estimate

This command can be used to determine an estimate of the cell density of a small area of your tissue. Preliminary Population Estimate is also useful as a tool to help determine a first order estimate of the number of counting frames in each section to be sampled. This estimate is based on the counting frame size, the target number of cells, the target Coefficient of Error (CE), or the number of sections to be sampled, and the observed cell density of a small region of a representative section.

Using a counting frame

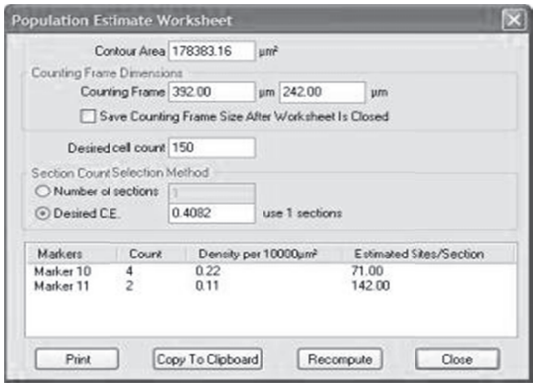
Before using the Fractionator and Optical Fractionator probes, you must determine how large a counting frame to use and how far apart the counting frames should be spaced.

To use the **Preliminary Population Estimate**, you will also need to decide how many sections to sample (or the target CE) and the desired target number of cells to count (250-400 is recommended). When this information is correctly determined for a given probe, the target number of cells will be counted when the probe is implemented and enough counting frames will be selected for each section to ensure a representative aerial sampling of each section. This should ensure that the CE for the study is within the desired range.

You must first define a counting frame size using Define Counting Frame and draw a closed contour around a relatively small representative region of one slide. A marker is then used to demarcate all cells in this small region, providing Stereo Investigator with a rough cell density measurement. The Population Estimate Worksheet can then be used to help balance the target cell count, the total number of sections (or target CE), the counting frame size and the number of counting frames per section.

To use Preliminary Population Estimate

1. After choosing **Probes>Preliminary Population Estimate,** and if there is more than one closed contour in the current data set, Stereo Investigator displays an aerial view of the entire tracing.
2. Click inside the closed contour that represents the region to be used for computing the preliminary population estimate.
3. Mark each cell in the representative area and choose **Probes>Preliminary Population Estimate** again. Stereo Investigator displays the **Population Estimate Worksheet** dialog box.



4. Type the **Number of sections** or the **Desired CE.**
5. If the estimated number of counting frames per section appears to be too low, or too high, you can modify the **Desired Cell Count, Desired CE, Number of sections**, or **Counting Frame Size**

and then click **Recompute**. Repeat this process until a reasonable number of counting frames has been achieved. Generally 10 counting frames per section is the target number. Note the final value for Counting Frame Size. This size is automatically saved and entered into any subsequent fractionator setup if the **Save Counting Frame Size After Worksheet is Closed** box is checked.

Preview SRS Layout

A useful feature of Stereo Investigator is the ability to preview a fractionator scan before it is actually implemented. The following procedure helps you see how Stereo Investigator performs the scan, allowing you to visually determine how the sampling parameters will work without actually performing a scan.

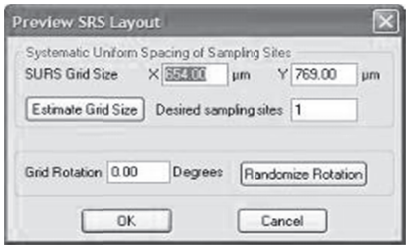
If there is more than one closed contour in the current tracing, Stereo Investigator displays an aerial view of the data. You may select the appropriate contour in which to perform the probe by clicking inside that contour.

Exclude Nested Contours

To exclude nested contours, click **Options>Stereology Preferences** and select the **SRS** tab. Check the **Exclude Nested Contours In Fractionator Probes** box. When Preview SRS Layout is selected, all contours are shown in the aerial view. If a contour is selected that contains other closed contours completely within its boundaries, the interior of those contours will be excluded from the probe layout.

To preview an SRS Layout

1. Click **Probes>Preview SRS Layout**
2. Click on a point. Stereo Investigator displays the **Preview SRS Layout** dialog box.



The SURS (Systematic Uniform Random Sampling) Grid size represents the distance in X and Y between adjacent counting frames, and therefore must be at least the size of the counting frame.

3. Enter the values for the X and Y SURS grid size. Alternatively, you may enter a value in the Desired sampling sites field, then click on the **Estimate Grid Size** button. This method will assign best guess estimates to the scan grid size values. These values are determined by the size and shape of the selected closed contour. They will most likely need to be manually revised. Typically most people find that it is desirable to use round numbers for the scan grid size, i.e. 250 instead of 244.54. If you know the size of the grid to be used, do not use the Estimate Grid Size button or the **Desired sampling sites** field—these are only intended to help you estimate an appropriate grid size in the initial setup of a sampling run.
4. Enter the **Grid Rotation** if desired, or use the **Randomize Rotation** button to choose a random degree of rotation. Stereo Investigator will display the entire region to be scanned, with an overlay of the scan grid lattice, and the locations of all of the counting frames.
   Stereo Investigator warns you that this preview is only intended for use in generating the initial

design of your sampling plan. Once the parameters have been determined, do not use the preview to select an arrangement of sampling sites to fit your tissue. Such selection removes an important random sampling step from the stereological process, and will introduce bias in your analysis.

5. If the preview shows that you have too many or too few sampling sites, make changes to the parameters so that the appropriate number of sites are included in your tissue. However, if the preview shows that the sites will fall on a location in your tissue in which you feel there will not be representative sampling, do not start another preview to get a better layout. Accepting the random placement of the sampling sites is a key component to systematic random sampling and the stereological principals.
6. Click the mouse anywhere in the tracing area or press the ESC key to exit preview mode. If you want to execute the probe displayed in the preview, you can then choose the appropriate probe name from the Probes menu to begin the probe.

You can use **Preview SRS Layout** while a fractionator probe is in progress to see an overview of the status of the probe. If you select **Preview SRS Layout** while a fractionator probe is running, an aerial display of the current probe is shown with the current counting frame blinking.

Optical Fractionator Workflow

Starts the Workflow for the Optical Fractionator. Each step in the workflow includes context-sensitive help to assist you. For more information, please see Introducing Workflows .

Space Balls Workflow

Starts the Workflow for Space Balls. Each step in the workflow includes context-sensitive help to assist you. For more information, please see Introducing Workflows.

Physical Fractionator Workflow

Starts the Workflow for the Physical Fractionator. Each step in the workflow includes context-sensitive help to assist you. For more information, please see Introducing Workflows.

Connectivity Assay Workflow

Starts the Workflow for Connectivity Assay. Each step in the workflow includes context-sensitive help to assist you. For more information, please see Stereo Investigator Workflows on page **Error! Bookmark not defined.**.

Fractionator

The **Fractionator** probe samples a 2D region of interest. The fractionator is a design based stereological method for systematic sampling of a region of interest. It is typically employed to sample populations that are too large to count exhaustively. The fractionator is most often used for two purposes.

- To estimate population counts and population density. The fractionator sampling method can be combined with a disector probe. This probe is commonly known as the physical disector. The fractionator sampling method can be combined with an optical disector probe. This probe is commonly known as the **Optical Fractionator**.

- To select random distribution of objects to analyze with local probes, i.e., the nucleator. The key features of the fractionator technique are that it is unbiased and it is efficient.

In certain circumstances the **Fractionator** probe can be used when it does not make sense to use an **Optical Fractionator** or **Optical Disector**. A good example of this is when counting plated cells for tissue culture analysis.

Prerequisites

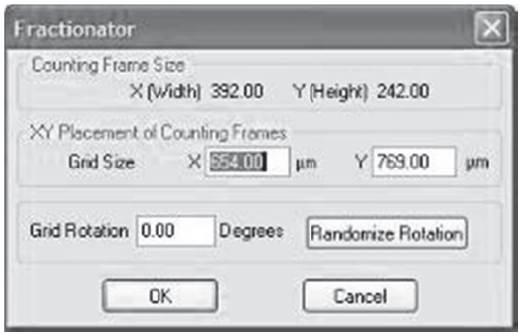
Before using the Fractionator, you will need to do some work beforehand. Use this checklist to help you get set up:

- Define the counting frame size using **Probes>Define Counting Frame**.
- Draw a closed contour around the region of interest that you want to sample.
- Determine the distance between counting frame sites (the XY Grid Size) using **Probes>Preview SRS Layout**.

Now you are ready to start the probe.

To use the Fractionator probe

1. Click **Probes>Fractionator**. Stereo Investigator displays the **Fractionator** dialog box



   with counting frame size and previously determined **XY Placement of Counting Frame** displayed. After you have confirmed the X and Y grid sizes, click **OK**.

2. **Stereo Investigator** automatically moves the microscope stage to the first sampling site. This first position is determined randomly.
3. Select a marker and mark all particles falling within the counting frame. See Counting Rules for details.
4. To move to the next sampling site, click **Next Scan Site** on the **Main** toolbar or use the **F2** key.

If you want to end a **Fractionator** probe before it is completed, choose **Probes>Fractionator** at any time during the scan.

When you have visited all of the sampling sites, the probe is complete. Click **Probes>Display Probes Run List** to see the results. For more information on the display and interpretation of results, see Display Probes Run List.

Right-click Options

When running a **Fractionator** probe, the following right-click menu options are available:

- **Standard Marker Options:** See Editing Markers.
- **Next Scan Site:** Moves to the next site in the Fractionator scan.
- **Preview Fractionator:** Displays the overview of the placement of counting frames within the selected contour. The current counting frame flashes. Click anywhere in the preview to return to the current counting frame.

Optical Fractionator

The **Optical Fractionator** probe lets you sample a 3D region of interest and estimate the total number of particles.

The optical fractionator is an important development in probes involving the estimation of population size. The optical fractionator is a combination of counting with the optical disector and counting with the fractionator. This combination, referred to in the literature as the optical fractionator (West, et al., 1991), has a number of practical attributes, such as being unaffected by tissue shrinkage and not requiring rigorous definitions of structural boundaries. The optical fractionator involves counting objects with optical disectors in a uniform systematic sample that constitutes a known fraction of the volume of the region being analyzed. In practice, this is accomplished by systematically sampling a known fraction of the section thickness, of a known fraction of sectional area, of a known fraction of the sections that contain the region of interest.

You use the optical fractionator to perform systematic sampling of populations distributed within a series of serial sections to estimate the population number in a volume. Properly designed systematic sampling yields unbiased estimates of population number. The theory underlying this sampling methodology also makes it possible to estimate the precision of the population size estimate for a single subject; this estimate of precision is called the **Coefficient of Error (CE).**

This CE should not be confused with the CE of estimates of the populations of the set of subjects that constitutes an entire experiment; the precision of population size estimates that are derived from a set of subjects is discussed later.

If you are running an Optical Fractionator on a FLUOVIEW system, please see Acquire SRS Image Series and Acquire SRS Image Stack Series on page 259 for instructions.

Two-stage systematic random sampling

The optical fractionator uses **two-stage** systematic sampling. In two stage systematic sampling two random number selections are made.

The first stage is the selection of which sections within a series to sample. The sequence of sections that spans the volume of interest is identified, taking care that the first and last sections in the sequence contain some of the specimen. Since only a subset of the series will be sampled, a section increment must be chosen, for example, suppose every 10th section will be sampled. The random choice of the initial section to sample (i.e. one of the first 10 sections) is made using random number selection. Multiples of the scanning interval (10 in this example) are then added to this random number to determine which individual sections are to be examined. For instance, if we start with the 3rd section, then the 13th, 2rd, 33rd, etc. sections would be sampled.

The second stage is to select a series of equally spaced optical disectors within each section and to only count cells that lie within them. To do this, exactly as with the fractionator probe, a region of interest must be delineated, a counting frame defined, and the distance between the counting frames in the XY plane must be determined. Then, just as with the optical disector, dimensions of a counting frame and top and bottom guard zones must be defined.

What are the design parameters for an optical fractionator probe run?

When designing an optical fractionator probe, the following factors will need to be considered. This section discusses why each of these factors is important. The following section, How do I implement an optical fractionator probe run? It gives you the details for how to actually determine values for these parameters.

Section Thickness

Stereo Investigator uses two types of section thickness. The first, **Cut Thickness** (also called **Block Advance**), is the full thickness of the tissue as it was in the intact tissue.

The second type is the **Mounted Thickness** of the tissue. This is the actual physical thickness of the tissue on the slide following processing and associated shrinkage. The actual physical thickness of the sections to be sampled should be determined empirically. You can use Stereo Investigator to do this by determining the difference between the focal depth of the top and bottom of the tissue. Section thickness will affect the counting frame thickness and the size of the guard zones.

It is recommended that the mounted thickness be no less than fifteen microns, with twenty to thirty microns being optimal for an Optical Fractionator scan. The cut thickness needed to obtain this mounted thickness depends on how the tissue is processed.

An optimal section thickness allows for an optical disector with four to five distinct optical planes through it. If you have a very high numerical aperture 100X Oil lens you can expect to have distinct optical planes every 0.5–0.7 microns. At lower magnification or with lower NA lenses, the optical sections (depth of field) will be thicker.

If sections are too thin, there may not be enough room for adequate guard zones, or the objective lens to be used may not have an adequately thin depth of field for this protocol.

If sections are too thick, it may be very hard to focus clearly to a given depth due to visibility constraints, working distances of objective lenses, etc. There may also be issues of consistent staining throughout the section thickness. Determining optimal thickness for an optical fractionator is an important part of setting up a study, and should be thoroughly evaluated before beginning a study.

Section Increment

There are several methods of determining an appropriate section increment. This value is obviously constrained by the total number of sections in the series that contain some portion of the volume of interest. It is desirable to sample as few sections as possible. It is also desirable to make sure that you are sampling enough sections to be seeing a representative cross sectional volume of the region of interest.

Normally, you would not want to sample every section, nor would you want to sample only one or two sections.

Starting Section

Once the section increment has been determined, the starting section index should be chosen at random for optical fractionator run. Stereo Investigator lets you do this easily when you define the first section of a series.

Counting Frame Size

The size of the counting frame should be such that it is small enough to fit in a single field of view when using the objective lens that will be used to count cells. Initially it should be large enough to contain, on average, one to six cells.

Counting Frame Distribution

In general, counting frames should be far enough apart that they cover only a fractional area of the region of interest, yet they should be distributed effectively over this region. This implies a reasonable number of counting frames per section.

Counting Frame Thickness

The thickness of the counting frame, added to the thickness of the upper and lower guard zones, must be less than or equal to the minimum physical section thickness. Thus, the maximum thickness of the counting frame is constrained by the section thickness (and guard zone thickness), while the minimum counting frame thickness is determined by the cell distribution and the depth of field of the objective lens that will be used to count the cells. This latter factor is important, since only cells that come into focus while focusing down through the counting frame will be counted. This implies that the depth of field of the objective lens should be significantly less than the thickness of the counting frame if possible.

Guard Zone Size

The guard zones should normally be thick enough to ensure that the tissue sampled by the 3D counting frame was not disturbed by the process of preparing the sections. The minimum guard zone thickness required will depend upon how the tissue was sectioned, as well as how it was processed before and after sectioning (e.g., guard zones may be unnecessary for plastic-embedded sections).

Prerequisites for an Optical Fractionator Probe Run

Before using the Optical Fractionator probe to estimate a population using a series of sections from a single animal, you need to set or specify a number of parameters. Then, you implement the probe across this series of sections. This section discusses how to determine these parameters, evaluate them, and then estimate the population using this probe. It then discusses how to acquire, evaluate, and compare estimates for groups of animals.

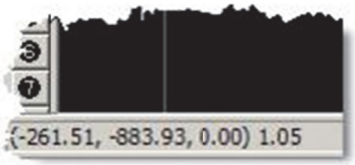
Measuring Section Thickness

It is important to know the minimum actual section thickness in order to properly determine the thickness of the counting frames and their guard zones. Most histological processes result in shrinkage along the Z-axis of a section (due to greater exposed surface area). Some cutting procedures result in inconsistent section thickness. Even if a section starts out with a consistent section thickness, different areas within the section can shrink differently, depending on the characteristics of the tissue. As a result of these problems the true physical section thickness usually does not know *a priori* with any accuracy.

You can use Stereo Investigator to determine the true section thickness by measuring the section thickness at several points in several sections to determine the variation in section thickness.

To measure the section thickness

1. Click the **Focus Position Meter** button to display the **Focus Position Meter.**
2. Focus at the top of a section using a lens that has a depth of field significantly smaller than the section thickness. If you cannot focus on the top of the section, try to focus on the first object below the top of the section. If there is not an object at the top of the section focus on the extracellular matrix at the top of the section. This can be difficult to execute, but it is essential for an accurate determination of section thickness.
3. Press **ALT+S**. Stereo Investigator sets the Z position to 0.
4. Focus to the bottom of the section. If you cannot identify the bottom of the section, try to adjust the stage height such that the bottom of the lowest object in the field of view is just leaving focus as you are focusing down through the section.
5. Look at the Z (focal) depth value provided by Stereo Investigator in the lower-left of the status pane.



This will be the third of the three coordinate numbers displayed in the parentheses. This value is the distance, in microns, between the top and bottom focal planes you have chosen. If you focused at the top and bottom of the section, it is the section thickness at this particular location.

Determining the Target Sample Count

You will need to sample enough cells to obtain a reliable estimate for the total population number. The uncertainty of an estimate is quantified by the Coefficient of Error, or CE. We will eventually evaluate the CE of an optical fractionator pilot study to determine if we have chosen appropriate parameters, one of which is the target sample count. Choosing a target sample count between 100 and 600 is often recommended in the stereology literature.

Determining the Counting Frame Size

It is recommended that an initial counting frame size be chosen such that it will fit entirely into a single field of view (when using the objective lens used to count cells) and large enough to contain approximately from 5 to 8 cells per counting frame.

Determining the Total Number of Sampling Sites

Ideally, one would like to start with perhaps 10 or more counting frames per section. We assume here that this should provide an adequate sampling resolution based on the consistency of cell distributions in the region of interest on each section. If cells are clumped together in small volumes of the region of interest more sampling sites might be necessary.

The following examples illustrate how the estimated sampling site count might be evaluated. These examples assume a target sample count of 200, and 100 sections, of which only a small number will be sampled.

Too Many Sampling Sites

Assume, for example, that the total number of estimated sampling sites shown on the worksheet is 600. In this case we will either count too many cells (for instance if we see two cells per sampling site), or we will visit a lot of sampling sites where we do not see any cells.

If the estimated cell density just determined is representative of the entire region of interest over all the sections to be sampled, this is a warning that we probably want fewer sampling sites. The counting frame size and the target sample count can be changed in the **Population Estimate Worksheet.** Click **Recompute** to view the resulting estimated number of sampling sites. Repeat this procedure until the number of sampling sites appears more reasonable.

Too Few Sampling Sites

Assume, for a different case, that the total number of estimated sampling sites shown on the worksheet is 10. In this case we will both count too many cells per counting frame (i.e. 20 on average), and we will not have enough counting frames to distribute over multiple sections (we are looking for about 10 counting frames per section initially).

Reasonable Number of Sampling Sites

If the total number of estimated sampling sites shown on the worksheet is 150, we might judge this to be adequate because, if we count one cell per counting frame on average, we could have 10 counting frames per section and sample 15 sections.

This provides us with very few, if any, empty counting frames, reasonable coverage of each section (e.g. 10 counting frames) and reasonable coverage of the total volume (i.e. 15 out of 100 sections).

These parameters may seem adequate, but they may not prove to be optimal for a full blown stereological survey, so we will still need to test them empirically.

Determining the Section Increment

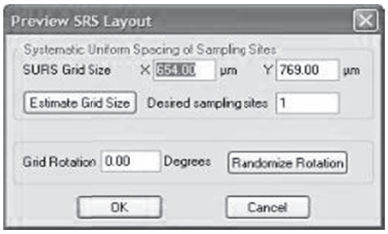
The next step is to select a section increment, the interval between sections that will be sampled.

In our example, we have settled on a counting frame size and target sample count that will yield approximately 150 counting frames. Since we have decided we want about 10 counting frames per section, we will need to sample 15 out of the 100 sections, i.e. about every 6th section (we divide 100 by 15 and the result is approximately 6.6). Thus, we have now determined an initial value of 6 for our Section Increment value.

Determining the Counting Frame Distribution

In order to determine the proper distribution of counting frames, use the following procedure until the desired number of counting frames (approximately 10 in our current example) are shown over the region of interest:

1. Pick a representative slide and draw a closed contour around the region of interest.
2. Click **Probes>Preview SRS Layout.** Stereo Investigator displays the **Preview SRS Layout** dialog box.



If there is more than one closed contour you will need to click inside the contour drawn around the region of interest.

3. Select appropriate values for the **SURS Grid Size** X and Y values. This is the distance between counting frames. Click **Estimate Grid Size** to generate the appropriate grid size based on the number of sampling sites you want within the contour. The SURS grid size values can then be adjusted manually if you want them to be round numbers.
4. Click **OK**. Stereo Investigator displays an aerial view of the contour with a pattern of counting frames overlaying it. The total number of counting frames is determined by the size and shape of the selected closed contour, the size of the counting frames and their distance from each other. The total number of counting frames for this combination of parameters is shown in the lower right status pane of the Stereo Investigator window. If the total number of counting frames is too large, for instance much larger than 10 for our example, you will need to use larger values for the **SURS Grid Size** X and Y values. If there are not enough counting frames you will need to use smaller values.
5. Click anywhere in the tracing window to dismiss the aerial preview display.
6. Repeat this process until the scan grid sizes are set so that you get approximately the correct number of counting frames distributed over the region of interest.

Stereo Investigator shows you a different layout for each preview because it uses a new random starting point for the grid placement. Remember that the layout that is previewed is never the layout that is used in an actual stereological run. Different numbers of sampling sites can end up on different previews, even when the spacing is the same. Use the preview to get a general sense of how many counting frames in the XY grid will fall on your region of interest, and then adjust the grid settings accordingly.

Determining the Optical Disector Height

The height of the optical disector needs to be viewed in light of several considerations:

- The thickness of the optical disector must be less than the thinnest portion of any section.
- It must be thick enough to provide a representative volume.
- It is helpful for it to be significantly thicker than the depth of field for the objective lens that is used when counting cells.

For instance, if sections were cut at 40 microns and the final section thickness varies between 18 and 20 microns, the optical disector height could be 12 microns allowing the top and bottom guard zones to be 3 microns.

Determining the Guard Zone Thickness

Determination of guard zone thickness is an important aspect in making sure the sampling procedure is unbiased.

Issues to consider are:

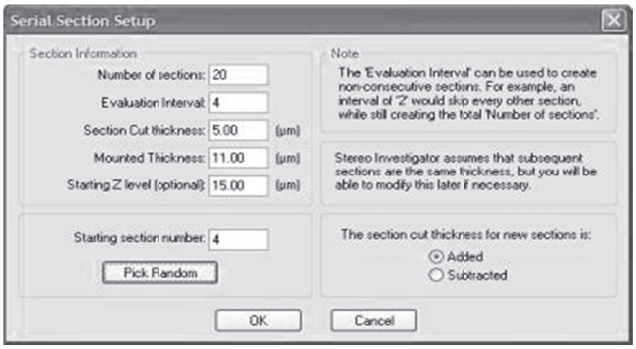
- They should be thick enough to ensure that the volume sampled by each 3D counting frame is representative. Common sources of bias that occur near the surface of a section are tearing of tissue, lost caps, etc.
- Their maximum size (assuming they are of equal thickness) is the minimum section thickness minus the thickness of the optical disector divided by 2.
- If portions of cells were plucked out of the tissue during sectioning, guard zones should normally be larger than the portion of the these cells lost from the surface of the section. If the sectioning process reliably cuts large cells this constraint may not be necessary.

How do I implement an Optical Fractionator probe run?

The procedure explained here describes how a full Optical Fractionator survey is to be performed. However, after initial values for all the parameters have been established, the same procedure should be used to perform a quick test that will allow you to refine the parameters. Plan to go through one of your specimens with a full Optical Fractionator survey as part of a pilot study determining the best parameters.

First, set up the Serial Section Manager

1. Place a representative slide under the microscope and navigate to the region of interest using a low magnification lens.
2. Before tracing the region of interest, click **Tools>Serial Section Manager**. Click **New Section** to define the parameters of your sections. Stereo Investigator displays the **Serial Section Setup** dialog box.



3. Enter the block advance (cut section thickness) and the mounted section thickness as determined by focusing at the top and bottom of the section described in **What are the design parameters for an optical fractionator probe run?** If the mounted section thickness is incorrect, it can be adjusted at a later time.
4. Enter the section evaluation interval, and if desired, you can use **Pick Random Starting Section** to choose a starting section for you.
5. The Z axis value for the top of the first section is 0 by default, but can be changed if you are starting at a known depth.
6. The section name is automatically entered as if all sections through the region of interest were present on the slide. According to the example shown above, the sections will be numbered 5, 13, 21, 29, etc.... (every 8th section starting with section 5). The section name can also be changed manually.

7. Click **OK** when all information has been entered

Set up the Optical Fractionator

8. Select a low magnification lens. Be sure to also select from the lens selection list on the toolbar. Draw a contour around the region of interest in which you want to count cells.
9. Switch to a high power objective lens suitable for counting cells; remember to select this lens from the list on the Stereo Investigator toolbar.
10. Select a marker for counting cells.
11. Click **Probes>Optical Fractionator**.

If you are using the Optical Fractionator to count multiple types of cells, use a different marker type to identify each cell type.

We recommend that you elect Refocus to top of section at each grid site and measure the section thickness at every site. This is the only way an accurate mean section thickness will be determined.

12. Choose the type of scan that you would like to perform:
    - Manual Focus
    - Automatic Focus – Scan Up
    - Automatic Focus – Scan Down
13. Under **Measure Thickness** select whether you want to measure the section thickness at each selected grid site and type the evaluation interval at every *nth* site. If you have very uneven tissue thickness within each section, it is recommended to check **Measure section thickness at each selected grid site**. With this option, you are instructed to focus at the top and bottom of the section at each scan site. These thicknesses are recorded and used to report **Number Weighted Section Thickness** results, which take into account the varying thickness in calculating your final estimated total.

Start the Optical Fractionator Scan

14. Click **OK** in the **Optical Fractionator** dialog box. Stereo Investigator randomly selects the starting point of the scan and moves the stage to the first counting frame. A random starting point is necessary to insure unbiased sampling.
15. Stereo Investigator displays the **Focus Top of Section** dialog box.

16. Follow the instructions in the dialog box.

17. If you have selected **Measure section thickness at each selected grid site** in the **Optical Fractionator** dialog box, Stereo Investigator displays the **Focus Bottom of Section** dialog box.

18. Follow the instructions in the dialog box. Stereo Investigator automatically moves the stage to the top of the counting frame. If the focus position meter is enabled, you will see the Z-axis extent of the counting frame in green. When the focal plane is above or below the counting frame height, the cursor is shown as an objective lens with a slash through it to indicate that cells should not be counted at the current focal plane.

19. Count cells within the 3D counting frame by placing markers on them. For information on selecting cells refer to the counting rules outlined in the section The Optical Disector. Focus through the extent of the 3D counting frame to count all cells.

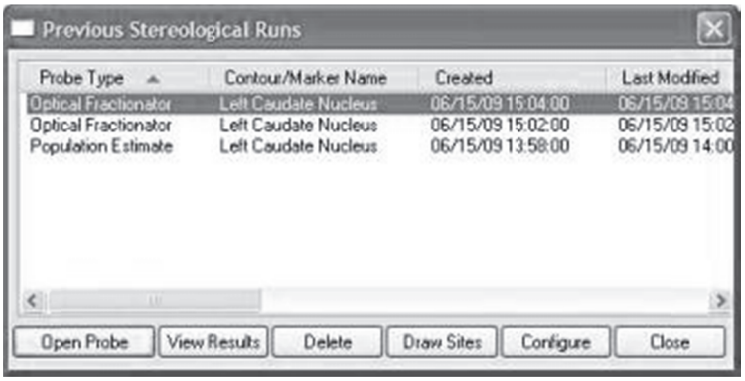
20. After counting all the cells according to the appropriate counting rules, click the **Next Scan Site** button to move to the next scan site
 -or-
Right-click and choose **Next Scan Site**
 -or-
Press F2.

Saving the data can be done at any time. Saving the data often, or at least after each section is analyzed, is highly recommended.

19. Repeat until you have examined all of the counting frames distributed across the region of interest for this section.

20. Click **Probes>Optical Fractionator** to end the run.

21. Click **Probes>Display Probe Run List**. Stereo Investigator displays the **Previous Stereological Runs** dialog box.



22. Check to make sure that the number of cells counted is close to, or somewhat larger than, the target number of cells (for a single section). To see the number of markers counted, right-click on

the marker toolbar and choose Show Marker Summary -or- choose Probes>Display Probe Run List and then choose the most recent Optical Fractionator run, and click View Results.

If the number of cells is not close to the target number for a single section, you may want to adjust the sampling parameters before completing sampling of the entire specimen. See the section below on Adjusting the Counting Frame Size and Distribution.

Right-click Options

When running an Optical Fractionator probe, the following right-click menu options are available:

- **Standard Marker Options:** See Editing Markers.
- **Next Scan Site:** Moves to the next site in the **Fractionator** scan.
- **Previous Scan Site:** Moves to the previous site.
- **Change Focus Method:** Displays the **Adjust Focus Method** dialog box.
- **Preview Optical Fractionator:** Displays the overview of the placement of counting frames within the selected contour. The current counting frame flashes. Click anywhere in the preview to return to the current counting frame.
- **Reset Top of Section/Measure Thickness:** If the section thickness was measured incorrectly, use this option to begin the measurement again, or to reset the section top.

Tracing subsequent sections

1. Return to a low magnification objective lens and move the stage so that a new section is visible, and then click the **Create New Section** button to define a new section in the **Serial Section Manager.** The values from the previous section are used to automatically enter section thickness, Z-depth and section name for the next section, but these values can be edited if necessary.
2. Trace a contour around the region of interest in the new section. The contour name should match the one used in the previous section.
3. Switch to a high power lens for counting, and click **Probes>Optical Fractionator**. Stereo Investigator displays the **Optical Fractionator** dialog box with the parameters used in the first section. These values should not be changed for a new section in the same specimen.
4. Click **OK** to begin counting the current section.

The stage is moved to the location of the first counting frame. Continue as above for the remaining sections of this specimen.

The counting parameters do not need to be the same between specimens, or between different regions of interest within a specimen, but for a given region of interest they must be kept the same for every section in a given specimen in order to extract valid CE data.

Adjusting the Counting Frame Size and Distribution

If the number of cells counted is significantly lower than the target cell count for a single section, then either the counting frame size should be made larger, or the distance between the counting frames needs to be made smaller (a smaller grid size resulting in more counting frames per section).

If counting frames in which cells were counted tended to have more than 10 cells on average, smaller counting frames may be needed. If many counting frames had none, or too few, cells, the size of the counting frame may need to be increased.

Decreasing the scan grid size (i.e. the distance between counting frames) will result in more counting frames for a given region of interest.

Increasing the counting frame size may yield more cells per counting frame.

Adjust the counting frame size and/or the scan grid size and perform the above test again. The goal here is to determine a set of parameters that will yield the desired target cell count after a full optical fractionator survey has been completed over a series of sections, but without having to actually perform a full survey.

In a full optical fractionator survey across a series of sections the distance between counting frames does not vary from one section to the next. However, since the region of interest in each section may vary in size, some sections can have more sampling sites than other sections. At this point we are only interested in making sure we count roughly the expected number of cells in the representative section.

How do I see the results of my probe run?

After you have performed at least one optical fractionator probe, you can view the results. Click **Probes>Display Probe Run List**. To view the results of a single probe, click on the name of the probe that you want to review and then click **View Results**. Stereo Investigator calculates the estimated total population number, as well as the estimated precision (i.e. Coefficient of Error, Variance Due to Noise, and SRS variance, etc.). To see the formulae used to generate these statistics, click the **Equations** button.

A very powerful feature of Stereo Investigator allows you to view the combined statistics for multiple surveys (within the same specimen). In this way, you can review the composite statistics for optical fractionator runs over a series of sections to achieve a total population estimate for an entire specimen. To do this, use the **SHIFT** or **CTRL** key together with the mouse to select the appropriate section surveys from the list. The selected surveys are highlighted. Choose **View Results** to see the combined statistics for the entire group of optical fractionator surveys. These results apply for the entire series of sections that have been sampled.

Evaluating the results of the probe run

After selecting one or more runs and clicking **View Results**, Stereo Investigator displays a dialog box containing the experimental parameters and results of the scan(s). The first heading in the dialog box is Parameters. Headings are found at the left side of the Sampling Results window.

Parameters

- **Data File Name:** This field displays the file name associated with this data set, if the data has been saved to, or read from, a file.
- **Date and Time:** This field displays the data and time when the probe was completed
- **Region:** This field shows the name of the contour type that defines the region of interest over which the probe was implemented. If a composite of several runs is being examined the contour name used for the first run is shown.
- **Number of Sampling Sites:** The number of sampling sites visited on all selected sections is shown here.
- **Contour Area:** This is the area of the contour bounding the regions of interest, or if multiple optical fractionator runs are being examined, the sum of the areas. Stereo Investigator calculates this area from the geometry of the bounding contour(s). This area can be compared with the area determined using the Cavalieri Probe.

- **Counting Frame Area:** The area of a single counting frame.
- **Counting Frame Thickness:** The thickness of the counting frames along the Z-axis.
- **Counting Frame Volume:** The volume of a single counting frame.
- **Counting Frame Width:** The X-axis width of each counting frame, in microns.
- **Counting Frame Height:** The Y-axis height of each counting frame, in microns.
- **Sampling Grid Width:** This represents the distance, along the X-axis, between counting frames (sampling sites).
- **Sampling Grid Height:** This represents the distance, along the Y-axis, between counting frames (sampling sites).
- **Sampling Grid Area:** This is the area of the region that is associated with each sampling step.
- **Section Thickness:** The value used for section thickness across all sections that were sampled. This should be the minimum actual section thickness as measured by Stereo Investigator.
- **Number Weighted Mean Section Thickness:** The number weighted mean of all sections measured by focusing at the top and bottom of the section. Check that this value is relatively close to the Section Thickness. More information about the number weighted thickness is found by clicking on the **Number Weighted Details** heading in the left panel.

Marker Counts

Marker counts and estimates of total number are found by clicking on the name of the marker of choice in the left panel of the Sampling Results window.

The total number of each marker is provided here. Normally each marker is used to represent one cell type. If an optical fractionator is being used to count multiple types of cells, a different marker type should be used to identify each cell type. The totals for each marker type are displayed.

- **Estimated Total by Optical Fractionator:** This field shows the estimated population count determined by the selected series of optical fractionator runs.
- **Est. Total by Number Weighted Section Thickness:** This shows the estimated population count determined by the selected series of optical fractionator runs, using for the section thickness value the number weighted section thickness.
- **Total Markers Counted:** This is the actual number of markers of this type that were counted during the optical fractionator survey.
- **Counts by Site:** This is the raw data of the number of counts for each counting frame visited in each of the runs of the optical fractionator.
- **Coefficient of Error Calculations**: Are described in more detail in Using Coefficient of Error.

Other Results

- **Z Depth Histogram:** Shows the Z depth location for markers placed in each section.
- **Planimetry:** These values show how area and volume are calculated based on the area contained within the traced contour. Variance Details are also provided.

Planimetry is not an unbiased estimate.

Interpretation of Results

Stereo Investigator provides four methods for calculating the Optical Fractionator Results for each type of marker used. The results vary based on the measurement used for the mounted (or post-processing) section thickness. The mounted section thickness value is divided into the counting frame thickness (or disector height) to calculate the height sampling fraction (*hsf*). Choose the results method to report the measurement that best reflects the histological properties of the region of interest.

Estimated Total by Optical Fractionator

This value is calculated using a single user-defined measurement value for the post-processing or "mounted" section thickness. This value is typically entered in Step 5 of the Optical Fractionator Workflow. However, it can also be altered after the counting procedure, by clicking **Edit Mounted Thickness** in the results window.

This is the only method calculated if you did not measure the section thickness while counting. Because this estimate is generated with only one value for the section thickness, local variations in section thickness are not accounted for. Due to this, it should be considered the least accurate of the 4 available methods that calculate the estimated totals in most situations. If section thickness variations do not occur such as with certain embedding protocols like plastic embedding, choosing to report the Estimated Total by Optical Fractionator is an acceptable estimated result. A historical note, this is also the method that was used in the original Optical Fractionator publications.

If you did not manually enter a value for the section thickness measurement (Step 5), this estimate will equal zero.

Estimated Total by Mean Measured Thickness

In contrast to the first reported option, Estimated Total by Mean Measured Thickness is calculated using section thicknesses measured during the counting process. When the option is chosen to measure while counting (Step 5 of the Optical Fractionator Workflow), the number of measurements used to calculate this estimate is based on the interval selected (e.g., if 2 is entered, then at every other counting site you would have been prompted to set the top and bottom of the section). Each counting site's measured thickness is averaged to produce a mean measured thickness value for the *hsf*.

Because this estimate is generated from the mean of all obtained section thickness measurements, it is considered to be the most accurate estimate of the region of interest when measurements are not performed at every site. You may also choose to use this value if you have a low frequency event (such as Brdu+ neurons) where you have many counting sites that contain zero objects, but still want to generate an accurate measured section thickness measurement for the *hsf* using a systematic interval for site measurement.

If you did not manually measure the section thickness while counting, this estimate will not be calculated.

Estimated Total by Mean Measured Thickness from Sites with Counts

This estimate is a variation of Estimated Total by Mean Measured Thickness. It is calculated using only the section thickness measurements made at counting sites that contain marked objects. Thus, no section thickness measurements from counting sites that have no objects are included in the calculated average. In many cases, this calculation will be nearly identical to Estimated Total by Mean Measured Thickness. By viewing the measurements per site on the Counts by Site tab, it is possible to see the contribution of measurements obtained from sites where there are zero counts. You may choose to use this value if you

chose to ignore measuring the section thickness where there were no objects or if you made errors in section thickness measurement that were not corrected when there were no objects to be marked.

If you did not manually measure the section thickness while counting, this estimate will not be calculated.

Estimated Total by Number Weighted Section Thickness

When the section thickness varies dramatically across the sections that include the region of interest, the fourth and final estimate can be reported. Estimated Total by Number Weighted Section Thickness is calculated using only the section thickness measurements from counting sites that contain markers. These measured thicknesses are then weighted by the number of objects associated with them to produce a weighted average. This number weighted mean section thickness is reported on the parameters tab and is used to calculate the height sampling fraction. The result should only be chosen if the thickness was measured at every sampling site.

If you did not manually measure the section thickness while counting, this estimate will not be calculated.

References

- Geiser, M., L.M.Cruz-Orive, and I.M. Hof (1990) Assessment of particle retention and clearance in the intrapulmonary conducting airways of hamster lungs with the fractionator. Journal of Microscopy 160 (Pt 1): 75-88.
- Glaser, E.M. and P.D. Wilson (1998) The coefficient of error of optical fractionator population size estimates: a computer simulation comparing three probes. Journal of Microscopy. 192(Pt 2): 163-171.
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- Gundersen, H.J.G. and E.B.V. Jensen (1987) The efficiency of systematic sampling in stereology and its prediction. Journal of Microscopy. 147(Pt3): 229-263.
- Schmitz, C. and P. Hof (2000) Recommendations for straightforward and rigorous methods of counting neurons based on a computer simulation approach. Journal of Clinical Neuroanatomy. 20:93-114.
- West, M.J., L. Slomianka, and H.J.G. Gundersen (1991) Unbiased stereological estimation of the total number of neurons in the subdivisions of the rat hippocampus using the Optical Fractionator. *The Anatomical Record* 231: 482-497.

L-Cycloid Optical Fractionator

This probe is used to estimate the ratio of total line length to unit volume within an Optical Fractionator sampling scheme.

Sets of curves (cycloids) are displayed in a grid pattern over the material to be examined. Markers are placed where these cycloids cross the projected lineal features of interest and the intersection event falls within the Optical Fractionator counting frame.

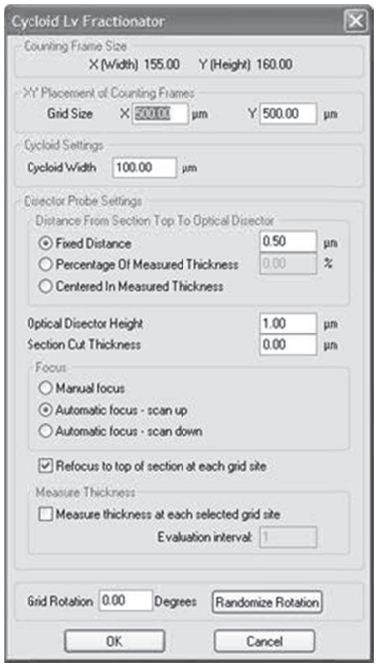
Prerequisites

- Vertical Uniform Random sections, or vertical slices of constant thickness having a random rotation around vertical axis

- Identifiable Vertical Axis visible in every section
- Lineal structure to be sampled must have a very small cross-sectional area or have a single one-dimensional lineal feature that can be identified (e.g.: a medial axis)

To perform an L-Cycloid Optical Fractionator probe run

1. At low magnification, perform a tracing of the area that you want to scan.
2. Switch to a lens that is suitable for viewing the linear structures.
3. Click **Probes>L-Cycloid Optical Fractionator**.
4. Click within the Tracing Window. Stereo Investigator displays the Cycloid Lv Fractionator dialog box.



5. This dialog box is almost identical to the setup for the Optical Fractionator, with the addition of the **Cycloid Width** field.
6. Enter a value for the cycloid width that allows for 2-4 intersections between the cycloid and the lineal feature of interest per counting frame.
7. Define the height of the counting frame, section thickness and top guard zone thickness.
8. Click **OK**. Stereo Investigator places you at the first sampling site and prompts you to focus at the top of the section.
9. Focus at the top of the section, and then click **OK**. Stereo Investigator automatically focuses down from the top of the section to the top of the counting frame that you defined in step 6, and overlays the test grid of cycloids on the slide image.
10. Right-click and select **Define Vertical Axis**. Use the mouse to grab the head of the arrow and move it so the long axis is aligned with the vertical axis of the tissue. If you are unable to determine the vertical axis at high magnification you can switch to a low magnification objective for this step.

11. Right-click and choose **Accept** when finished defining the vertical axis.
12. Select a marker for counting intersections
13. Use the left mouse button to mark wherever the cycloid curves cross the lineal features of interest within the counting frame. Mark intersections in all focal planes of the tissue that fall within the Z-boundaries of the counting frame.
14. When all intersections in a given counting frame have been marked, move to the next counting frame by right-clicking and choosing **Next Scan Site**, pressing **F2**, or using the **Next Scan Site** button.

To stop before all sites have been visited, click **Probes>L-Cycloid Optical Fractionator** again.

View Results

Stereo Investigator displays the **Sampling Results** dialog box after all sites have been visited. You can also see the results by clicking **Probes>Display Probe Run List**, and selecting **L-Cycloid Optical Fractionator** runs of interest in the list and then clicking **View Results**.

The **Parameters** pane shows the experimental parameters used in the sampling runs.

The **Planimetry** pane of the results shows the area and volume estimates.

References

- Stocks, E.A., McArthur, J.C., Griffen, J.W., and P.R. Mouton (1996). An unbiased method for estimation of total epidermal nerve fibre length. Journal of Neurocytology 25: 637-644.

IUR Planes Optical Fractionator

This probe is used to estimate the total line length within an Optical Fractionator sampling scheme when **Isotropic Sections** are available. If isotropic sections are not available, the **Isotropic Virtual Planes** or **Space Balls** probes should be used.

Sets of parallel lines are displayed over the material to be examined. These lines represent planes that extend along the Z axis. Markers are placed where the lineal features of interest intersect the planes and the intersection event falls within the Optical Fractionator counting frame.

Prerequisites

- Thick IUR slices (i.e. Isotropic Sections)
- Lineal structure to be sampled (e.g., fibers) must have a very small cross-sectional area or have a single one-dimensional lineal feature that can be identified (e.g.: a medial axis)

To perform an IUR Place Optical Fractionator probe run

1. Start the **Serial Section Manager** if multiple sections are to be sampled.
2. At high magnification, define the counting frame.
3. At low magnification, perform a tracing of the area that you want to scan.
4. Click **Probes>Preview SRS Layout** to set the desired spacing between counting frames.

5. Switch to a lens that is suitable for viewing the linear structures.

   Click **Probes>IUR Optical Fractionator**. Stereo Investigator displays the IUR Planes Fractionator dialog box.

6. This dialog box is almost identical to the setup for the **Optical Fractionator**, with the addition of the **IUR Planes Settings** field.

7. Enter a value for the **Spacing of Planes** that allows for 2-4 intersections between the planes and the lineal feature of interest per counting frame.

Angle of Sampling Planes: The angle of the planes can also be randomized or a predetermined angle entered. If no value is entered, the planes will appear as horizontal lines. The planes always extend straight down through the section, so the lines do not appear to move as you focus through the section.

8. Define the height of the counting frame, section thickness and top guard zone thickness.

9. Click **OK**. Stereo Investigator places you at the first sampling site and prompts you to focus at the top of the section.

10. Focus at the top of the section, and then click **OK**. Stereo Investigator automatically focuses down from the top of the section to the top of the counting frame and overlays the test grid of planes on the slide image.

11. Select a marker for counting intersections.

12. Use the left mouse button to mark wherever the planes (lines) cross the lineal features of interest within the counting frame. Mark intersections in all focal planes of the tissue that fall within the Z-boundaries of the counting frame.

Counting Rules: Count only lineal features that completely cross the displayed lines (or where the identifiable center of the lineal feature crosses the line). Do not count cases in which the line is tangent to the lineal feature. Count the intersection where the lineal feature is most clearly in focus. It is OK to count a lineal feature twice if it intersects the same line twice within the counting frame.

13. When all intersections in a given counting frame have been marked, move to the next counting frame by right-clicking and choosing **Next Scan Site**, or use the **Next Scan Site** button.

14. To stop before all sites have been visited, choose **IUR Planes Optical Fractionator** from the **Probe** menu. Stereo Investigator displays the **Sampling Results** dialog box after all sites have been visited.

View Results

Click **Probes>Display Probe Run List** and select all IUR Planes Optical Fractionator runs of interest in the list, and then click **View Results**.

The **Parameters** pane shows the experimental parameters used in the sampling runs.

The **Planimetry** pane of the results shows the area and volume measurements.

Formulas

$$est\ L = 2\frac{a}{l}\sum I \cdot \frac{1}{ssf} \cdot \frac{1}{asf} \cdot \frac{t}{h}$$

$$\frac{a}{l} = spacing$$

$$asf = \frac{area\ (Frame)}{are\ (x, y\ step)} = \frac{x_{cf} \cdot y_{cf}}{x_{step} \cdot y_{step}}$$

Symbols and References

$\frac{a}{l}$	area per unit of test line
$\sum I$	number of intersections
ssf	section sampling fraction
asf	area sampling fraction
t	section cut thickness
h	height of counting fraction

References

- Gokhale, A.M. (1990). Unbiased estimation of curve length in 3-D using vertical slices. J Microsc. 159, 133-141.

Area Fraction Fractionator Probe

The Area Fraction Fractionator (AFF) is essentially a Cavalieri point counting estimate of area and volume performed on a systematically selected fraction of the tissue. The probe is designed to estimate the fraction of a region occupied by a subregion. Any number of subregions may be examined using the AFF. An example of the use of the AFF would be where an investigator wants to compare the ratio of white matter to gray matter in the spinal cord. Another example would be where an investigator would like to know the ratio of muscle fiber to connective tissue to vasculature in a muscle.

This area fraction probe is implemented by performing a fractionator scan of the area of interest, while overlaying a rectangular lattice of points (as in the Cavalieri estimator). The user selects a marker to represent the object(s) or region(s) of interest and clicks on the grid points that both 1) fall on the objects or region of interest, and 2) are contained within the counting frame. The user then selects another marker to represent another region of interest, and clicks on the grid points that 1) fall in the region of interest and 2) are contained within the counting frame, but 3) do not fall on the objects of interest.

If it is desired to measure the area fraction of several types of objects, use additional marker types. Use one marker type to correspond to each type of object.

As with the Cavalieri probe, a shortcoming of the method is that there may be uncertainty in judging whether points near the boundaries of the area are inside or outside of the region of interest or object of interest.

Area Fraction Estimation

The area fraction, A_A is calculated for each type of marker used. Relative area fractions for each marker used are reported. Keep in mind that according to Delesse's theorem, the area fraction is equal to the volume fraction $A_A=V_V$.

Prerequisites and Design

The design of the Area Fraction Fractionator probe includes:

- The size of the counting frame.
- The distance between fractionator sampling sites.
- The appropriate distance between the points of the lattice point grid that will be used.

You will also need to know interval between the sections to be sampled.

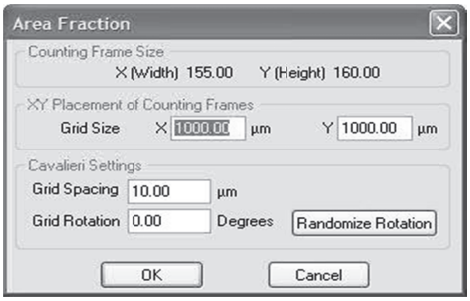
Here are some general guidelines for designing an area fraction probe:

- The fractinator's scan grid size should be set so that it yields an appropriate number of sampling sites on each section (e.g., between eight and ten). The number of sites should be large enough so that an even distribution of sites is obtained, yet not so large as to require exhaustive sampling.
- The counting frame should be sized so that it is large enough to distinguish between the regions of interest within the counting frame. A general rule of thumb is that the counting frame is 1/3-2/3 of the field of view used for viewing the objects of interest.
- The smaller the distance between lattice grid points, the more accurate the estimate. However, using too many points can be inefficient.

To perform an Area Fraction Fractionator probe run

1. Start Stereo Investigator and use the joystick to locate the first section to be sampled.
2. Click in the tracing window to place a reference point.
3. Start the **Serial Section Manager** if more than one section is going to be sampled.
4. Trace a region of interest using the contour mapping tool. This can be done at low magnification (i.e., with low precision), as the contour measurements are not used in the AFF calculations.

5. Switch to the magnification at which the analysis will be performed and define an appropriate counting frame size.
6. Click **Probes>Preview SRS Layout** to set the desired spacing between counting frames.
7. Click **Probes>Area Fraction Fractionator.**
8. If there is more than one closed contour outlining several regions, an aerial view of the entire tracing will appear, and you will need to click inside the region of interest. The **Area Fraction** dialog box is displayed.



You will see the Counting Frame size in the top of the Area Fraction dialog box, as well as the XY Placement of Counting Frames determined by Preview SRS Layout. The Cavalieri Settings determine the distance between grid points used to mark the extent of the area of interest. It is usually not necessary to rotate the grid in the AFF analysis, although a desired grid rotation can be entered, or the Randomize Rotation button used to select a random rotation for the grid.

9. When all variables have been entered, click **OK**. The stage moved to the first sampling site. The first site is a randomly selected location keyed off of the upper left corner of the region. A counting frame is displayed with a grid of intersection points arrayed on the screen.
10. Select a marker type to represent the object(s) of interest. Click on the grid points located within the counting frame that fall on the object(s) of interest. This places a marker on the grid intersections that you click on.

The Snap to Grid mode is available to place the markers directly on the grid intersections. Right-click and select Snap to Grid from the bottom of the right-click menu. Paint mode is the default Snap to Grid function, in which all grid intersections within the bounds of the circular cursor are marked when the cursor passes over them. If Snap to Grid mode is selected, new options appear on the right-click menu, including **Paint Mode, Erase Mode, Marquee Mode, Fill Mode** and **Replace Mode**. To return to the previous right-click menu, deselect Snap to Grid mode.

If your mouse has three buttons, click the middle mouse button to automatically switch to **Snap to Grid** and **Replace** modes. In these modes, all you need do is click the middle mouse button and then paint/fill the sub-region automatically.

11. If you would like to evaluate the area of several types of objects, select another marker type to represent the additional object(s) of interest. Click on the grid points located within the counting frame that fall on the additional object(s) of interest. This places a marker on the grid intersections that you click on.
12. Select a marker type to represent the area of the region of interest that is not occupied by the object(s) of interest. Click on the grid points that fall in the region of interest, which are contained within the counting frame, but do not fall on the objects of interest.

13. To go to the next site, click on the **Next Scan Site** button on the Main toolbar. If you want to go back to the previous scan site, **click Move>To Previous Scan Site**.
14. Repeat the previous step until the scan is completed.

Viewing Results

To view the results, click **Probes>Display Probe Run List**.

Select an **Area Fraction** probe entry from the list shown and then click **View Results**. If you chose more than one Area Fraction probe, specify the section periodicity, e.g. if you sampled every 4th slide, enter 4 here.

The results page lists the total number of objects or interest marked, the estimated area covered by those objects, and the area fraction filled by the objects of interest.

Formulas

$$\widehat{V_v}(Y,ref) = \frac{\sum_{i=1}^{m} P(Y)_i}{\sum_{i=1}^{m} P(ref)_i}$$

$$\hat{A} = \frac{1}{asf} \cdot a(p) \cdot P(Y_i)$$

Symbols and References

P(ref)	points hitting the reference volume
Y(Y)	points hitting the region
$\widehat{V_v}$	estimated volume fraction
asf	area sampling fraction
A(p)	area associated with a point
$\hat{A}$	estimated area

Grid Spacing

The grid spacing is equal in both axes of the sampling grid. The square of the grid is *a(p).*

References

- Howard CV and Reed MG "Unbiased Stereology" Bios Scientific Publishers, Oxford 1998.

Nearest Neighbor

The **Nearest Neighbor** analysis is a method to investigate the 3D spatial arrangement of objects such as cells within thick sections of the tissue of interest. X-Y-Z coordinates of "parent cells" are recorded, as well as the coordinates of all of the neighboring "offspring cells" close to each parent cell. From this coordinate data, the nearest neighbor distance is calculated for each parent cell, as are the nearest neighbor distributions for the region of interest.

The **Nearest Neighbor** analysis is performed within the context of a systematic random sampling of the tissue; as such, a counting frame size and XY grid spacing between counting frames is determined for each specimen. Once the **Nearest Neighbor** probe is started, the software drives the stage to each counting frame location and displays a standard counting frame. Within each counting frame, the parent cells are marked, and then a scan is performed through the entire Z-depth of the counting frame, marking each offspring cell that is close to the parent cell. The shortest X-Y-Z distance is calculated to determine the closest offspring cell. Any other parent cells in the counting frame are then marked and the process is repeated until all parent cells are marked.

An Optical Fractionator analysis is automatically performed on the parent cell population, so an estimated total number of the parent cell population for the region of interest is generated with the Nearest Neighbor results

Prerequisites

The setup for a Nearest Neighbor survey is essentially the same as that for an Optical Fractionator. Therefore, you should be thoroughly familiar with the Optical Fractionator principles and setup before beginning a Nearest Neighbor analysis. For more information, see the Optical Fractionator.

How do I perform a Nearest Neighbor probe run?

Set up the Serial Section Manager

1. Place a representative slide under the microscope and navigate to the region of interest using a low magnification lens.
2. Before tracing the region of interest, start the **Serial Section Manager**. Click on New Section to define the parameters of your sections. Stereo Investigator displays the Serial Section Setup dialog box.
3. Enter the block advance (cut section thickness) and the mounted section thickness as determined by focusing at the top and bottom of the section. If the mounted section thickness is incorrect, it can be adjusted at a later time.
4. Enter the section evaluation interval, and if desired, you can use the Pick Random Starting Section button to choose a starting section for you.
5. The Z axis value for the top of the first section is automatically calculated, but can be changed if you are starting at a known depth.
6. The section name is automatically entered as if all sections through the region of interest were present on the slide. According to the example shown above, the sections will be numbered 5, 13, 21, 29, etc., (every 8th section starting with section 5). The section name can also be changed manually.
7. Click **OK** when all information has been entered

Determine the Counting Frame Size

1. Select a high magnification lens that will be used do the survey. The lens should be a high enough magnification to be able to clearly distinguish individual cells. It is convenient if the lens is also sufficiently low power to show each cell's nearest neighbor in the same field of view. It is not required that the nearest neighbor be in the same field of view, but this will save significant time in the survey.

2. Click **Probes>Define Counting Frame** and set the size and location of the counting frame. Remember that it is often most convenient to set the rough size of the counting frame graphically (by dragging the edges of the counting frame) and then round the numbers in the dialog box for simplicity in reporting.

Determining the Counting Frame Distribution

In order to determine the proper distribution of counting frames, use the following procedure until the desired number of counting frames (approximately 10 in our current example) are shown over the region of interest:

1. Pick a representative slide and draw a closed contour around the region of interest using a low magnification lens.
2. Click **Probes>Preview SRS Layout.** Stereo Investigator displays the **Preview SRS Layout** dialog box.
3. Select appropriate values for the X and Y SURS Grid Size. This is the distance between counting frames. You can use the Estimate Grid Size button to generate the appropriate grid size based on the number of sampling sites you want within the contour. The SRS grid size values can then be adjusted manually if you want them to be round numbers. Values can also be entered manually, but if this is done, do not then also click the Estimate Grid Size button, as this will replace the entered values with a generated estimate.
4. Click **OK**. Stereo Investigator displays an aerial view of the contour with a pattern of counting frames overlaying it. If there is more than one closed contour you will need to click inside the contour drawn around the region of interest.
5. The total number of counting frames is determined by the size and shape of the selected closed contour, the size of the counting frames and their distance from each other. The total number of counting frames for this combination of parameters is shown in the lower right status pane of the Stereo Investigator window.
6. If the total number of counting frames is too large, for instance much larger than 10 for our example, you will need to use larger values for the X and Y SURS Grid Size. If there are not enough counting frames you will need to use smaller values.
7. Click anywhere in the tracing window to dismiss the aerial preview display.
8. Repeat this process until the scan grid sizes are set so that you get approximately the correct number of counting frames distributed over the region of interest.

Each time the Preview is run, a different layout is presented. This is because a new random starting point for the grid placement is used. Do not repeat the Preview until you find a layout that pleases you. This introduces bias. Use the preview to get a general sense of how many counting frames in the XY grid will fall on your region of interest, and then adjust the grid settings accordingly.

Set up the Nearest Neighbor Probe

1. Select a low magnification lens. Be sure to select from the lens selection list on the toolbar. Draw a contour around the region of interest in which you want to analyze distribution (this may have already been done in determining the counting frame placement with **Preview SRS Layout**).
2. Switch to a high power objective lens suitable for counting cells (the one used to define the counting frame size); remember to select this lens from the list on the Stereo Investigator toolbar.

3. Select a marker for counting cells.
4. Click **Probes>Nearest Neighbor**. Stereo Investigator displays the **Nearest Neighbor Probe** dialog box.

Values determined during the Preview SRS Layout operation, including the Counting Frame Size are already entered for you. You can make any changes if needed.

5. Enter the guard zone height in the **Distance From Section Top to Optical Disector** field. This can either be a fixed distance or a **Percentage of Measured Thickness**. Remember that *[((Guard Zone height)x2) + Optical Disector Height]* must be less than or equal to the minimum **Mounted Section Thickness**.
6. Choose the type of scan that you would like to perform, **Manual Focus, Automatic Focus—Scan Up** or **Automatic Focus—Scan Down.**
7. Under **Section Thickness Measurement Settings**, select whether you want Stereo Investigator to measure the section thickness for you, and how frequently those measurements should be made. If you have very uneven tissue thickness within each section, it is recommended to check Measure section thickness at each selected grid site. With this option, you are instructed to focus at the top and bottom of the section at each scan site. These thickness are recorded and used to report Number Weighted Section Thickness results, which take into account the varying thickness in calculating your final estimated total.

Start the Nearest Neighbor Scan

1. Click **OK** in the **Nearest Neighbor Probe** dialog box. Stereo Investigator randomly selects the starting point of the scan and moves the stage to the first counting frame. A random starting point is necessary to insure unbiased sampling.
2. Stereo Investigator displays the **Focus Top of Section** dialog box. Do this, and then click **OK**.
3. If you have selected Measure section thickness at each selected grid site in the Optical Fractionator dialog box, Stereo Investigator displays the **Focus Bottom of Section dialog** box. Do this, and then click **OK**. Stereo Investigator automatically moves the stage to the top of the counting frame. If the focus position meter is enabled, you will see the Z-axis extent of the counting frame in green. When the cursor is above or below the counting frame height, the cursor is shown as an objective

lens with a slash through it to indicate that cells should not be counted outside of the counting frame height.

4. Select the first parent cell within the 3D counting frame by placing a marker on it.
5. The display of the counting frame is then turned off. Focus through the full depth of the tissue and click to place a marker on all offspring cells that are in proximity to the selected parent cell. The same marker type can be used for parent and offspring cells.
6. As each offspring cell is clicked, a dashed white circle is drawn around the parent cell with a radius of the distance to the nearest offspring cell. If a new offspring cell is further away from the parent cell than a previously marked offspring, the radius of the circle does not change. Note that the radius of the circle changes as you focus up and down, representing a sphere around the parent cell. The offspring cells do not need to fall within the counting frame, which is why the counting frame is hidden during this step.
7. Once all cells in proximity to the parent cell have been marked, right-click and choose **Next Parent Cell**. The counting frame is now displayed, and another parent cell can be selected.
8. All cells that fall within the 3D counting frame should be defined as parent cells. If two parent cells are offspring cells of one another, the circle drawn around the second parent cell is automatically drawn with a radius of the distance to the first parent cell.
9. Once all parent cells have been defined and their offspring cells marked, the analysis is complete for that counting frame. Click the **Next Scan Site** button to move to the next scan site, right-click and choose **Next Scan Site,** or press **F2**.
10. Repeat steps 4 through 9 until you have examined all of the counting frames distributed across the region of interest for this section.
11. Check to make sure that the number of cells counted is close to, or somewhat larger than, the target number of cells (for a single section). To see the number of markers counted, choose Probes>Display Probe Run List and then choose the most recent Nearest Neighbor run. Note that on the marker toolbar all parent and offspring cells are included in the marker total. However, in the Probe Results, only the parent cells are used to generate the Estimated Total numbers.

Tracing subsequent sections

1. Return to a low magnification objective lens and move the stage so that a new section is visible, and then click the New Section button to define a new section in the **Serial Section Manager**. The values from the first section are used to automatically enter section thickness, Z-depth and section name for the next section, but these values can be edited if necessary.
2. Trace a contour around the region of interest in the new section.
3. Switch to a high power lens for counting, and click **Probes>Nearest Neighbor**. Stereo Investigator displays the **Nearest Neighbor** dialog box with the parameters used in the first section. These values should not be changed for a new section in the same specimen.
4. Click **OK** to begin counting the current section.
5. The stage is moved to the location of the first counting frame. Continue as above for the remaining sections of this specimen.

The counting parameters do not need to be the same between specimens, but must be kept the same for every section in a given specimen in order to extract valid CE data.

Results and interpretation

After selecting one or more runs and clicking the **View Results** button Stereo Investigator displays the **Sampling Results** dialog box. The first heading shown in the dialog box is **General Results**. Headings are found at the left side of the Sampling Results window. The basic results and Marker totals are the same as found in **Optical Fractionator** results.

General Results

The general results pane reports the experimental parameters of the study. On this pane you will find information about the counting frame size and placement, the number of sampling sites, and section information.

- **Data File Name:** This field displays the file name associated with this data set if the data has been saved to or read from a file.
- **Date and Time:** This field displays the data and time when the probe was completed.
- **Region:** This field shows the name of the contour type that defines the region of interest over which the probe was implemented. If a composite of several runs is being examined the contour name used for the first run is shown.
- **Number of Sampling Sites:** The number of sampling sites visited on all selected sections is shown here.
- **Contour Area:** This is the area of the contour bounding the regions of interest, or if multiple optical fractionator runs are being examined, the sum of the areas. Stereo Investigator calculates this area from the geometry of the bounding contour(s). This area can be compared with the area determined using the Cavalieri Probe.
- **Counting Frame Area:** The area of a single counting frame.
- **Counting Frame Thickness:** The thickness of the counting frames along the Z-axis.
- **Counting Frame Volume:** The volume of a single counting frame.
- **Counting Frame Width:** The X-axis width of each counting frame, in microns.
- **Counting Frame Height:** The Y-axis height of each counting frame, in microns.
- **Sampling Grid Width:** This represents the distance, along the X-axis, between counting frames (sampling sites).
- **Sampling Grid Height:** This represents the distance, along the Y-axis, between counting frames (sampling sites).
- **Sampling Grid Area:** This is the area of the region that is associated with each sampling step.
- **Section Thickness:** The value used for section thickness across all sections that were sampled. This should be the minimum actual section thickness as measured by Stereo Investigator.
- **Number Weighted Mean Section Thickness:** The number weighted mean of all sections measured by focusing at the top and bottom of the section. Check that this value is relatively close to the **Section Thickness**.

More information about the number weighted thickness is found by clicking on the **Number Weighted Details** heading in the left panel.

Marker Counts

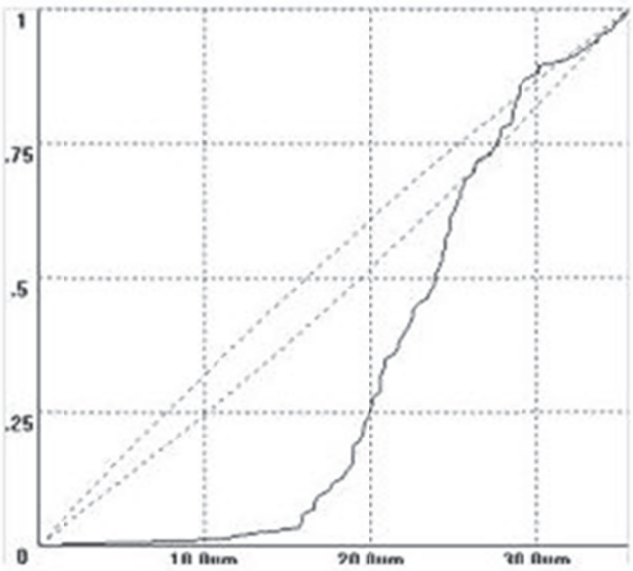
In the marker counts pane, you will find the estimated total by optical fractionator for each marker type used.

Marker counts and estimates of total number are found by clicking on the name of the marker of choice in the left panel of the **Sampling Results** window.

The total number of each marker is provided here. Normally each marker is used to represent one cell type. If an optical fractionator is being used to count multiple types of cells, a different marker type should be used to identify each cell type. The totals for each marker type are displayed.

- **Estimated Total by Optical Fractionator:** This field shows the estimated population count determined by the selected series of optical fractionator runs.
- **Est. Total by Number Weighted Section Thickness:** This shows the estimated population count determined by the selected series of optical fractionator runs, using for the section thickness value the number weighted section thickness.
- **Total Markers Counted:** This is the actual number of markers of this type that were counted during the optical fractionator survey. These markers also represent the cells designated as parent cells during the Nearest Neighbor survey.
- **Counts by Site:** This is the raw data of the number of counts for each counting frame visited in each of the runs of the optical fractionator.
- **Sampled Distances:** This is the raw data of the nearest neighbor distance for each designated parent cell. The X,Y,Z distance from each parent cell to all designated offspring cells is calculated, and the shortest of these distances reported for each cell
- **CRF Graph:** The G-graph is a graphical representation of the degree of dispersal or aggregation of the cell population in question. This is a type of empirical distribution function graph used to provide information on the type of spatial arrangement of particles within a given reference system. The graph is derived as follows: The CRF-graph (cumulative relative frequency) is created from the raw data you have measured, the nearest neighbor distances (nnd). For each nnd on the X-axis the graph shows a Y-value that indicates the probability that any particle would be that distance or less from another particle.

Example CRF Graph and Explanation



In this example of a CRF-graph the nearest neighbor distances are given on the x-axis. For this discussion, ignore the area delineated by the dotted lines. If you look at a given **nnd**, you can tell the probability that any particle would be that distance, or less (since it's a cumulative frequency graph), from another particle. You can use the CRF graph to tell something about the three dimensional arrangement of your particles. For instance, in this graph, there is a *scarcity* of short distances that indicates a relatively dispersed situation. If we compared this data set to another group of cells that were more clustered, the CRF would rise more rapidly because there are many short distances. For examples where CRF graphs of **nnds** were compared please see Fig. 2 in Schmitz, etal. Altered Spatial Arrangements…..2002. or Fig. 4 of Segal et al., Spatial distribution and density…, 2008. Please be careful about comparing CRF graphs that come from systems with different number of cells and/or different densities.

- **CRF Details:** This table shows the probability that a cell anywhere in the tissue will fall within a given distance of another cell (will have a particular nearest neighbor distance). The maximum distance shows a probability of one, since all cells will be within that distance or less of their nearest neighbor.

References

- Diggle (1983). Statistical analysis of spatial point patterns. London, Academic Press.
- Schmitz C, G. N., Hof PR, Boehringer R, Glaser J, Korr H. (2002). Altered spatial arrangement of layer V pyramidal cells in the mouse brain following prenatal low-dose X-irradiation. A stereological study using a novel three-dimensional analysis method to estimate the nearest neighbor distance distributions of cells in thick sections." Cereb Cortex 12(9): 954-960.
- Segal D, S. C., Hof PR. (2008). Spatial distribution and density of oligodendrocytes in the cingulum bundle are unaltered in schizophrenia. Acta Neuropathol 117(4): 385-394.

Isotropic Virtual Planes

Isotropic Virtual Planes is a length estimator used when IUR or VUR sections are not practical. Length can be determined for many different types of objects: tubules, nerve fibers, small blood vessels, microvilli, etc.

As in other length estimators, the probe is a set of parallel planes, and the counted parameter is the number of profiles that transect each plane within a defined counting region. The difference is that the planes are set at an isotropic angle to the tissue, emulating an IUR sectioning orientation.

Note that length measurements cannot be corrected for shrinkage, so the length reported is the final length in the processed tissue.

A series of sites are selected within each section by systematic random sampling. At each site, a 3D sampling box of constant volume is superimposed upon the slide to represent the boundaries of the counting area.

The sampling box is set up using the Define Counting Frame command, but is not technically a counting frame with rules of inclusion and exclusion as seen in other probes. The inclusion and exclusion zones are defined by the virtual planes.

A series of equidistant and parallel virtual planes with a random start are used to virtually section the 3D sampling box. The planes are represented in the program as a series of active lines (also called "moving lines"), that move across the section as the focal plane changes. The speed and direction of the active line movement across the screen depend on the angle of the virtual plane with respect to the X-Y plane.

Prerequisites and Design

- Thick sections: Sections need to be significantly thicker than the diameter of the fibers to be measured, and thick enough for several distinct optical planes through the section. Use Quick Measure Line to measure the diameter of the tubules if you think they might be thicker than the section thickness after processing.
- The structure of interest must be stained through the depth of the tissue section. Unstained tissue must be transparent enough to see the stained structure throughout the depth of the section.
- Thin focal planes, which are achieved using a high magnification, high numerical aperture lens, generally oil immersion.
- A contour traced around the region of interest.

Before starting the probe, use **Probes>Define Counting Frame** to define a counting frame. Choose a size that includes several profiles of the fiber(s) on which the length estimation is to be performed. If you are working with acquired images or image stacks, the counting frame needs to be smaller than the image in order to avoid the bias of an edge effect.

Preview

Click **Probes>Preview SRS Layout** to preview the arrangement of sampling sites within the tissue section. The **Preview SRS Layout** dialog box allows for entry of grid size, automatic estimate of grid size for a given number of sampling sites, and grid rotation.

After selecting settings, Stereo Investigator displays the sampling site layout in the tracing window. Click in the tracing window to end the **Preview** mode.

Section thickness

It is useful to know the actual section thickness before beginning any probe. Use a high power objective lens with a high numerical aperture to focus at the top and bottom of a few sites throughout your sections to get a rough idea of the section thickness. This value can be edited later, but an approximate value is needed for setting up the 3D sampling boxes and guard zones.

Serial Section Setup

Typically you run this probe on more than one section for a given tissue sample, so the first step in running the probe is to set up the **Serial Section Manager**. Use the **Serial Section Setup** dialog box to enter the cut thickness, actual post-processing section thickness, and section periodicity.

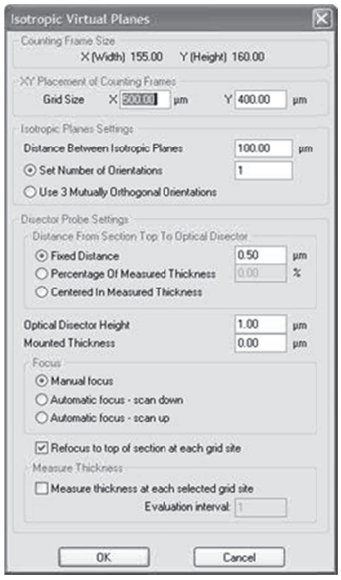
Isotropic Virtual Planes—Counting rules

- Only count intersections that lie within the bounds of the 3D sampling box (drawn in white by the software).
- Each time a fiber cross section (referred to as a 'profile') is transected by the green active line (representing the isotropic virtual plane), a marker is placed on the point of intersection to count the profile.
- Do not count locations where the fiber merely touches the active line, the line must fully transect the fiber.
- For each profile intersection marked, follow the fiber through its full visible extent in current focal plane. If the fiber is continuously transected in the same focal plane by the active line until it runs into the exclusion zone (where the line is red), the original profile should not be counted.
- If the profile is transected by the green line, leaves the path of the active line, then is transected by the red exclusion line, the profile transected by the green line should be counted.
- If the fiber is transected by the green line in one focal plane, and then by the red line in another focal plane, it should still be counted -- unless the fiber intersection with the moving line stays in focus until the intersection moves to the red region of the line.
- If a fiber is transected multiple times by any one active line, but moves away from the line between focal planes, it should be counted at each intersection.

To help visualize this, remember that the fiber moving in and out of focus as it crosses the active line represents the fiber moving in and out of the sampling plane. If the sampling plane were the only visible part of the tissue, the fiber would appear to be segmented, and each fraction would be counted.

To perform an Isotropic Virtual Planes probe run

1. Start the Serial Section Manager and define the first section.
2. At low magnification, draw a contour around the region of interest.
3. Switch to an appropriate high magnification, and then click **Probes>Isotropic Virtual Planes**. Stereo Investigator displays the **Isotropic Virtual Planes** dialog box.



- If an SRS preview was used, the **Scan Grid Size** determined in the preview is automatically entered in the **XY Placement of Counting Frames** field.
- **Isotropic Planes Settings:** Enter a distance in microns between the isotropic planes. This distance is determined by the density and size of the profiles. This distance should be small enough that all profiles in the sampling box are intersected by at least one line. Also enter the desired number of orientations per sampling site. The distance between planes can be optimized during the pilot study.

Multiple orientations per site increases the efficiency of the probe by analyzing many different orientations within few sampling boxes rather than analyzing many sampling boxes, each with a new orientation.

- **Distance from Section top to Optical Disector** is the top guard zone height. This should be larger than the average thickness of the profiles to be counted and/or average tissue damage depth. It can also be set to a percentage of the measured section thickness, in which case the user must measure the section thickness at every sampling site.
- **Sampling Box Height** should be the minimal section thickness minus the top and bottom guard zones.
- **Mounted Thickness** is the actual z-depth of the sections as mounted on the slides (not the cut thickness). This should be measured with your microscope before starting. This value can be edited when viewing results if the initial estimate proves incorrect.
- **Focus:** Choose manual focus. If Refocus to top of section at each sampling site is selected, the program prompts you to focus at the top before setting up the counting box at each sampling site. If Measure section thickness at each grid site is selected, the program prompts you to focus at the top of the section and bottom of the section at each sampling site (or at a subset of sites set with Measurement site periodicity).

When the probe is started, the software drives the stage to the first sampling site. The virtual planes are represented as lines that move as the focal plane is moved up and down. If the lines do not appear to move, that is because the virtual plane is orthogonal to the field of view; in essence you are looking down on the edge of the plane. When viewed within the sampling box depth, the

active line is green within the sampling box, red on one side of the box, and blue on the other. Green indicates the inclusion line, red is the extension exclusion line, and blue is a 'line of indifference' used to indicate the position of the virtual plane outside the sampling box.

When the stage moves to the first counting box, the cursor appears as a diagram of a microscope objective with a slash through it and a + sign. This symbol indicates that the plane of focus is outside the counting frame as it lies in the sampling plane area. The active line is also completely red to indicate that the focal plane is above the 3D counting region. Focus down until the cursor becomes a simple crosshair again and begin counting.

View Overview Layout: At any time during the probe run, right-click and choose **Preview Isotropic Planes** to see an overview of the probe layout. Stereo Investigator displays the full file with the sampling boxes overlaid on the tracing. The current sampling box is indicated by flashing. Click anywhere in the preview window to return to the current sampling site.

4. Select a marker to count transections.
5. Focus through the section until all intersections have been counted. More than one plane orientation can be used to count each sampling box. To move to the next orientation, right-click and choose **Next Layout** (which lists the layout number). The same sampling box is then resampled from a different orientation.
6. Once all orientations have been completed, right-click and choose **Next Scan Site**.

Artificial Edge: If the section contains an artificial edge (a cut tissue edge or the edge of an acquired image), right-click and select **Artificial Edge**. The site is eliminated from the results calculation, and all markers in that site are disregarded by the software. The edge of the field of view in a live image is not an artificial edge, since the stage can be moved to see objects beyond the current field of view.

7. Complete all sampling sites within the section. Move the stage to a new section, use the **Serial Section Manager** to designate a new section, and repeat the procedure.

View Results

Length and Length Density

This page displays the estimated length based on the sampled intersections. The results of the length estimate for the probe are listed here as **Estimated Lv** and **Estimated Total Length**. The parameters listed on this page are:

- **Sampling Fraction Product**
- **Total Corners inside Region:** The corners of the counting frames that fall within the contour of interest are used to calculate the total area for the Lv value.
- **Total Corners Possible:** The total corners possible is the number of counting frames multiplied by 4, the total corners of all counting frames.
- **Average Area of Planes inside Boxes:** The average area of the sampling planes contained within the counting frames.
- **Estimated Lv:** The length/volume estimate result.
- **Estimated Total Length:** The total length estimate for the entire volume sampled.

Section Details

This page of the results displays the sampling results grouped by section name. For each section, we provide the following information:

- **Average number of Markers (in the section)**
- **Total Corners in Section:** Again, the total in ROI corners of all of the counting frames.

Individual Runs

Under each section listing sampling details are displayed for each run performed on that section. Click on the name of the run to view sampling details that include:

- **Layouts in Probe:** The number of plane layouts used.
- **Corners in Probe:** The number of counting frame corners (as used in the volume calculation) that fall within the contour of interest.
- **Total Possible Corners:** The number of corners on all counting frames, whether they fall inside or outside the contour.
- **Fraction of Corners in Probe:** The fraction of the total possible corners that fall within the contour of interest.
- **Total Plane Area in Probe:** The total area of all of the sampling planes used.
- **Average Plane Area per Layout:** The total plane area divided by the number of plane layouts.
- **Average Plane Area Per Sampling Box:** The total plane area divided by the number of counting frames.
- **Total Markers for All Layouts**
- **Average Markers per Layout**

Separate pages are listed under each run providing more information about:

- **Markers:** Marker summary for each marker type, including number of markers counted in the run, and a breakdown of counts by site
- Plane Areas by site
- Layout Orientations

Click **Equations** to view the equations and formulae used in generating the results.

References

- Larsen JO, Gundersen HJG, Nielsen J (1998). Global spatial sampling with isotropic virtual planes: estimators of length density and total length in thick, arbitrarily orientated sections. Journal of Microscopy Vol 191, Pt3, pp. 238-248.

Space Balls

The **Space Balls** probe is used when IUR or VUR sections are not practical, and length estimations are desired. Length can be determined for many different types of objects: tubules, nerve fibers, small blood

vessels, surfaces, microvilli, etc. As a general term, the objects measured are referred to as fibers in this documentation.

In this length probe, the probe is a sphere or hemisphere virtually embedded in the tissue, and the counted parameter is the number of profiles that transect the edge of the sphere within a defined counting region. Since the surface of a sphere is isotropic, the need for IUR sections is eliminated.

Length measurements cannot be corrected for shrinkage, so the length measured is the actual length in the processed tissue.

A series of sites are selected within each section by systematic random sampling. At each site, a 3D sphere of constant volume is superimposed upon the slide to represent the intersection sites to be counted.

The item to be counted is a "profile", that is, the point where the fiber transects the sphere boundary. If fibers are thick, the user must identify the center point of the fiber, and only count locations where the center point transects the sphere outline.

Prerequisites

- Thick sections (significantly thicker than the diameter of the tubules/fibers to be measured)
- Structure of interest must be stained through the depth of the tissue section. Unstained tissue must be transparent enough to see the stained structure throughout the depth of the section.
- Thin focal planes achieved with a high magnification, high numerical aperture lens, generally oil immersion.
- A contour traced around the region of interest.

Typically this probe is run on more than one section for a given tissue sample, so the first step in running the probe is to set up the Serial Section Manager. Use the **Serial Section Setup** dialog box to enter the cut thickness, actual post-processing section thickness, and section periodicity.

Section thickness

It is useful to know the actual section thickness before beginning any probe. Use a high power objective lens with a high numerical aperture to focus at the top and bottom of a few sites throughout your sections to get a rough idea of the section thickness. This value can be edited later, but an approximate value is needed for setting up the 3D sampling boxes and guard zones.

Counting Frame

A counting frame does not need to be defined for the **Space Balls** probe. However, the previously defined counting frame size does have an influence on the grid size if the counting frame is larger than the desired grid. If **Preview SRS Layout** does not let you specify a large enough number of sampling sites, click **Probes>Define Counting Frame** to define a smaller counting frame.

Preview

Click **Probes>Preview SRS Layout** to preview the arrangement of sampling sites within the tissue section. If more than one contour is present in the current section, an overview of the data file is presented, and the desired contour should be selected. The **Preview SRS Layout** dialog box allows for entry of grid size, automatic estimate of grid size for a given number of sampling sites, and grid rotation.

Once the settings have been selected, the sampling site layout is shown in the tracing window. Click in the tracing window to end the **Preview** mode.

To perform a Space Balls probe run

1. Start the **Serial Section Manager** and define the first section.
2. Draw a contour around the region of interest.
3. Click **Probes>Preview SRS Layout** to set the size of the scan grid.
4. Click **Probes>Space Balls.** Stereo Investigator displays the **Space Balls** dialog box.

Probe settings are:

- If an **SRS Preview** was used, the **Scan Grid Size** determined in the preview is automatically **entered in the XY Placement of Counting Frames** field.
- **Space Balls Settings:** Enter a radius in microns for the sampling sphere. This radius should allow for 2-3 transection events per sampling site. The sphere or hemisphere must be sized to fit entirely within the tissue thickness, including top and bottom guard zones, if they are used.
- Select **Use Hemispheres** to use the top half of a sphere as the sampling probe. Counts that are on the bottom edge of the hemisphere are factored in as 1/2 counts in the results.
- **Distance from Section Top to Top of Sphere** is the top guard zone height. This should be larger than the average thickness of the fibers to be counted. It can also be set to a percentage of the measured section thickness, in which case the program automatically selects measurements of the section thickness at every sampling site.
- **Mounted Thickness** is the actual Z-depth of the sections as mounted on the slides (not cut thickness). This should be measured with your microscope before starting. If you are using the program to measure the section thickness at each sampling site, it is not necessary to enter a value for the mounted section thickness, as it is calculated by the program. This value can be edited when viewing results if the initial estimate proves incorrect.
- **Focus:** Choose **Manual Focus** or **Automatic focus - scan up** or **Automatic focus - scan down.** If **Refocus to top of section at each grid site** is selected, Stereo Investigator prompts you to focus at the top before setting up the counting box at each sampling site. If **Measure thickness at each selected grid site** is selected, the program prompts you to focus at the top of the section and bottom of the section at each sampling site (or at a

sample of sites set with Measurement site periodicity). Use If **Measure thickness at each selected grid site** for Number Weighted Thickness results.

View Overview Layout: At any time during the probe run, right-click **Preview Space Balls** to see an overview of the probe layout Stereo Investigator shows the full file with the sampling boxes overlaid on the tracing. The current sampling box is indicated by flashing. Click anywhere in the Preview window to return to the current sampling site.

5. When the probe is started, Stereo Investigator drives the stage to the first sampling site. When the stage moves to the first counting box, the cursor appears as a diagram of a microscope objective with a slash through it and a (+) sign. This symbol indicates that the plane of focus is above the counting frame as it lies in the sampling plane area. No probe is visible, as the top of the section is above the top of the sphere. No counting frame is shown. Focus down until a small circle is visible in the center of the field of view. If the grid spacing is small, multiple circles may be visible. Only mark the one in the center, as the other sites will be systematically visited.
6. Select a marker to count transections. Place a marker at each location where a fiber crosses the currently visible circle.
7. Focus through the section, marking each intersection between a fiber and the sphere, until all intersections have been counted. The circle will appear larger in each subsequent focal plane to represent the sphere or hemisphere.
    - Count as 1/2: This option is available in the right-click menu if Hemispheres are being used and the current focal plane is near the equator of the hemisphere. If a linear feature is at the widest point of the hemisphere, it should be counted as 1/2, since it would also be counted in the lower half of a full sphere.
8. Right-click and choose Next Scan Site.
9. Complete all sampling sites within the section. Move the stage to a new section, use the **Serial Section Manager** to designate a new section, and repeat steps 2-7.

Viewing Results

The Space Balls probe yields two types of results: Parameters lists the design parameters and measurements. Planimetry values show how area and volume are calculated based on the area. Variance Details are also provided.

Planimetry is not an unbiased estimate.

References

- Mouton PR, Gokhale AM, Ward NL, West MJ. Stereological length estimation using spherical probes. Journal of Microscopy Vol 206, Pt1, April 2002. pp. 54-64.

Petrimetrics

The Petrimetrics probe is used for 2D in vitro studies, such as estimating the length of fibers. It uses the Merz grid in its investigation.

The Petrimetrics Probe uses the curvilinear test system devised by Merz known as the Merz Coherent Test System.

A set of semicircles laid out in an alternating grid pattern is displayed over the material to be examined. Markers are placed wherever the test lines cross the boundaries of the particle that you want to estimate.

You may wish to review the Merz discussion for more details on the Merz probe.

Grids, Counting Frames, and Probe Layouts—How Do You Determine the Best Parameter Values to Use?

We're frequently asked about the "correct" or "recommended" settings for grids, counting frames, and probe layouts. The short answer is that there are no "golden numbers" to use when creating your study. Remember that your goal isn't to count every particle or measure every fiber—you are sampling to get an estimate. Here are some questions to ask when starting:

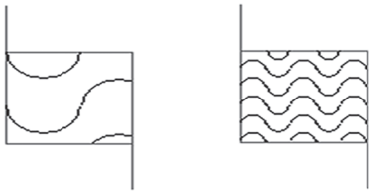
- **How good an estimate do I need?** For example, if your fiber length is uniformly long, parallel, and evenly spaced you could go with fewer sample sites, a larger Merz radius, and a larger counting frame and you would get a highly accurate estimate. Most samples aren't uniform and homogenous, however. The more non-homogenous your sample, the more your intersects will be less predictably oriented in space; therefore, you will need more sampling for your study to produce acceptable results. More sampling means more sampling sites, a smaller Merz radius, and smaller counting frames.
- **Are there any general guidelines, or "rules of thumb" to get me started?** We recommend at least ten counting frames per region of interest, and aim for 200 intersections per petri dish. Remember that these are just general guidelines to get you started.
- **Is there a Coefficient of Error (CE) I can use?** There is no CE for use with the Petrimetrics probe.

To use the Petrimetrics Probe

1. Load your image or data file.
2. Trace your area of interest.
3. Click **Probes>Define Counting Frame**.
   Stereo Investigator displays the **Define Counting Frame Numerically** dialog box.
4. Type the values in the dialog box, and then click **OK**.
   -or-
   Left-click and drag the mouse pointer in the main windows to graphically size the counting frame.

Right-click to change the counting frame colors or force it to be a square.

5. Click **Probes>Petrimetrics**.
   Stereo Investigator displays a warning dialog box.
6. Click **No**. You will not be using the **Serial Section Manager** since you are working on a 2D sample.
   Stereo Investigator displays the **Petrimetrics** dialog box.
7. Type any adjustments to the X and Y measurements for the **Grid Size**. This is the grid that the counting frames will be placed into for your work.
8. Type any adjustments to the **Merz Radius** settings. A larger number results in larger curvilinear lines spaced farther apart. A smaller number results in smaller curvilinear lines spaced closer together, as seen here.



The first counting frame has a Merz radius of 30µm; the second has a Merz radius of 10µm. (The counting frames in this illustration do not use the red/green you would see on screen.)

9. Type a figure for **Grid Rotation**, if desired, or click **Randomize Rotation**, if desired.
10. Click **OK**.
11. Select a marker to use for counting.

At this point you can begin counting. You can also click **Probes>Preview SRS Layout**.

12. Place a marker wherever the test line of the Merz lines touch a particle. When you are finished with the first site, click the **Next Scan Site** button, and place markers at the next site. Continue until **Stereo Investigator** lets you know that all sites have been visited.
13. Click **OK**, and then click **Display Probe Run** List.
14. Click **Open Probe** to view the image, tracing, and markers placed with the **Petrimetrics** probe.
15. Click **View Results** to view probe results. Highlight an entry in the left column to see the different results.

Further Reading

Howard, C.V. (2004) Unbiased Stereology (Advanced Methods) Oxford: Taylor and Francis Group.

Made to Order

Made to order layouts are available to users who are designing their own probes; for example, combining counting frames or systematic random sampling with other local and/or global probes. The data obtained from a **Made to Order** probe is in its raw form, and estimates and error estimations must be calculated manually. For users new to **Stereo Investigator**, and used to working with stereological tools such as acetate overlays, **Made to Order Layouts** will feel the most familiar. However, many of the custom probes that **Made to Order Layouts** would be used for can be generated by combining existing **Stereo Investigator** probes. Many of these probes can be easily integrated by running the probes concurrently, and then the results are automatically calculated with the relevant error calculations.

For instructions on how to set up **Made to Order Layouts**, see Define Made to Order Layouts.

To create a Made to Order layout

1. Define the layout using **Probes>Define Made to Order Layouts.**
2. Start the **Serial Section Manager** if multiple sections are to be used.
3. If a contour-bound region is to be used, map the contour and click **Probes>Preview SRS Layout** to determine the XY Grid Spacing.

4. Click **Probes>Made to Order.** The **Made to Order** dialog box is displayed.
5. Any previously determined Grid Size is entered, or a grid size can be entered at this time. Select a Probe Layout from the drop-down menu. In the Disector Probe Settings, set the guard zone height, focus method, and whether to use number weighted section thickness. Click **OK** when all fields have been entered.
6. **Stereo Investigator** automatically moves the microscope stage to the first sampling site. This first position is determined randomly.
7. Your custom probe layout is displayed.
8. Select a marker for counting events and mark the locations where the events or particles intersect your probe elements.
9. When all events in a given grid location have been marked, move to the next counting frame by right-clicking and selecting **Next Scan Site**, or use the **Next Scan Site button**.
10. To stop before all sites have been visited, deselect **Made to Order** from the **Probes** menu.

The results for **Made to Order** probe are presented in raw form, listing a number of markers placed in each scan site of the probe. Interpretation and calculation of final estimates is up to the user.

Cavalieri Estimator Probe

The Cavalieri Estimator probe estimates the area and volume of regions. The Cavalieri probe is a global probe.

The Cavalieri probe is implemented by overlaying a rectangular lattice of points on the area of interest and then counting the number of points in the lattice that lie within the area of interest.

A shortcoming of the method is that there may be uncertainty in judging whether points near the boundary of the area are inside or outside of the contour. The uncertainty is dependent upon how accurately you are able to judge the location of the contour that separates the area of interest from surrounding regions. A second shortcoming of this technique is that the precision estimations rely on a "shape factor" that is often obtained by an educated guess.

A more accurate estimation of the area of a region may be obtained by carefully tracing the region with the Stereo Investigator Contour Tracing Tool. However, if many sections are to be sampled, the Cavalieri is an extremely efficient method for obtaining a volume estimate.

This probe is used to estimate area and volume. The Cavalieri probe is implemented by overlaying a rectangular lattice of points on the area of interest and then counting the number of points in the lattice that lie within the area of interest.

What can I estimate with the Cavalieri Estimator probe?

Area Estimation

Both the estimated area of the region of interest, and an estimated CE for this estimate can be determined from the number of points counted and the known spacing between points.

Volume Estimation

Volume estimation of regions that are contained in serial sections entails, first of all, the estimation of the volume slices seen in the individual sections of the serial section sequence. An individual section's volume

is estimated as the cross sectional area of the region (as seen at the top or bottom of the section) multiplied by the thickness of the section. The total volume across multiple sections is the sum of all the individual section volumes.

When the number of sections in the sequence is large, this becomes an onerous task. Fortunately, it is usually not necessary to perform it; a systematic random sample of the sections is usually adequate enough to provide a volume estimate with the precision you desire.

Once you have estimated the areas of each section in the systematic sample, you will be able to calculate the estimated volume and its precision, given as a coefficient of error (CE). Stereo Investigator bases the volume calculation upon the distance between sections in the systematic samples. This distance must be constant throughout the sequence. The area estimates of multiple sections can be combined to give a total volume estimate and CE.

In order to estimate volume accurately, it is usually helpful to determine the original section thickness (immediately after cutting on the microtome) by actual measurement instead of relying on microtome settings. These thickness values can then be used for estimating the original volume of the region of interest if there has not been much shrinkage in the XY plane as the sections are prepared. If the actual thickness after histological preparation is used, the volume estimate will be that of the volume after any shrinkage that occurs during histological preparation. If the amount of shrinkage is known along the X, Y and Z axes then the original volume can also be estimated.

Prerequisites and Design

Decide upon an appropriate distance between the points of the point grid that will be overlaid on the region of interest. The smaller the distance between points, the more accurate the estimate.

For volume estimation you will also need to know either the thickness of the sections to be sampled, or the thickness interval between the sections to be sampled.

What is the Shape Factor and why is it important?

The shape factor is a key component of the formula used to compute the nugget effect. The nugget effect is used to calculate the coefficient of error.

The shape factor is the perimeter of the contour around the area of interest divided by the square root of the area. This value can be found in the **Contour Measurements** window, listed for each contour used. To display the **Contour Measurements** window, click **Display>Display Settings>Accessories** and check the box next to **Contour Measurements,** or use the **Contour Measurements** button on the toolbar.

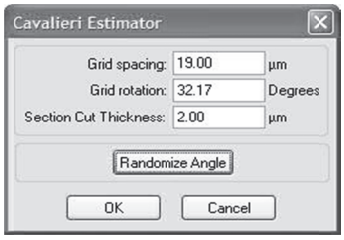
You may edit the appropriate shape factor for the region of interest that has been sampled. Click **Display>Where Is** to get an aerial view of the region of interest. You can click the **Edit Shape Factor** button in the **Sampling Results** dialog box to choose (judging by eye) a shape factor that most closely approximates that of the region of interest. After changing the shape factor those calculations that depend upon it are automatically updated.

When examining Cavalieri runs for a series of sections, a composite shape factor, perhaps an average of the shape factors of the individual contours, should be used.

To perform a Cavalieri Estimator probe run

This procedure is for a single section.

1. Click **Probes>Cavalieri Estimator**. Stereo Investigator displays the Cavalieri Estimator dialog box.



3. Enter the size of the grid spacing. This will determine the X and Y spacing between neighboring points in the array.
4. Optionally enter a rotation value for the placement of the grid or click **Randomize Angle**.
5. Enter the pre-shrinkage thickness of the tissue (the thickness of the tissue as it is cut) in **Section Cut Thickness** if you want to estimate volume with the Cavalieri Probe.
6. Click **OK.** Stereo Investigator displays a grid of small intersections (+) overlying the tissue.
7. Select a marker from the marker tool bar and click on each grid intersection "+" that lies inside the area of interest. There are 4 modes available for placing markers. The standard mode places a marker at the location of the cursor when the left button is clicked. The other modes use Snap to Grid mode, and all markers are placed on the grid intersection closest to the cursor at the time the left mouse button is clicked.

To enable the different modes of painting markers, first click **Options>Stereology Preferences**>**Cavalieri** tab. Check the box next to **Allow Snap to Grid for Cavalieri Probe**. Once this box is checked, the options listed here are enabled. If this box is not checked, markers are placed in exactly the location of the cursor when the left button is clicked.

8. With a marker selected, right-click in the tracing window to toggle between the different Snap to Grid options (found at the bottom of the right-click menu).
    - **Snap to Grid Mode:** In this mode, a marker is placed on the grid intersection that lies closest to the location of the cursor when the left-click button is pressed. Clicking on a grid intersection a second time erases the marker.
    - **Paint Cavalieri Markers Mode:** Uses the circular cursor as a paintbrush to "paint" markers whenever the cursor passes over a grid intersection. Adjust the size of the circular cursor using the "+" and "-" keys of the numerical keypad, or the mouse wheel. Hold down the left mouse button to paint markers.
    - **Erase Cavalieri Markers Mode:** Works just like the **Paint Cavalieri Markers Mode**, except that markers within the circular cursor are erased.
    - **Marquee Mode:** Allows you to draw a box around the grid intersections to be marked. Left-click at the top left of the area to be marked and then drag the cursor to the bottom right of the rectangular area while holding down the left mouse button. When the mouse button is released, markers are placed on all included grid intersections. Drawing the box in reverse (bottom right to top left), erases the enclosed markers.

While in Marquee mode, the option to **Paint Markers Into Contours** is available from the right-click menu. This option paints markers on every grid intersection included within a selected contour. If there is more than one contour, you are presented with an overview of the file and prompted to click in a contour to be marked. **Erase Markers In Contour** works the same way, but erases all markers in a selected contour.

If the object is larger than a single field of view, you may use any of the commands from the **Move** menu, or the arrow buttons on the status bar in order to view and mark all of the intersections within the object. If there is a contour drawn around the region of interest, a **Meander Scan** may be used during a Cavalieri Probe run to be sure all sites within the contour are seen.

9. After all of the intersections have been marked, click **Probes>Cavalieri Estimator** to end the probe run.

This information can be viewed later by choosing **Probes>Display Probe Run List**. Select a Cavalieri probe entry from the list shown and then click the **View Results** button.

The **Sampling Results** dialog box displays the estimated area and volume of the selected region and the estimated CE. You can experiment with different **Shape Factor** values and observe their effect on the CE.

To perform a volume estimation with multiple sections

1. Click **Tools>Serial Section Manager**.
2. Click **New**.
3. Enter the section thickness.
4. Enter the section interval that you want to sample.
5. Select a random first section manually or use the **Random Number Generator**.
6. A **Section Name** is automatically entered. You may use this name or replace this name with your own more meaningful name if you would like.
7. Click **Next**.
8. Click **Probes>Cavalieri Estimator**.
9. Start with the randomly selected initial section of the systematic sequence.
10. Follow the procedures described above under How do I perform a Cavalieri Estimator probe run?
11. After you have completed the first section, click **Tools>Serial Section Manager**, and then click **New**. Verify that the values shown are correct.
12. Prepare to view the new section indicated in the dialog box.
13. Select the option **Only Display Current Section**, and then click **OK**.
14. Repeat steps 10-13. Make sure you use the same marker from section to section.
15. Once you have used the Cavalieri Estimator on each section, click **Probes>Display Probe Run List**. Select all of the Cavalieri runs that are applicable using the SHIFT key or CTRL key together with the left mouse button.
16. Click **View Results**.

The **Sampling Results** dialog box displays the estimated area and volume of the selected region and the estimated CE. You can experiment with different **Shape Factor** values and observe their effect on the CE.

If you trace a closed contour around the region of interest, Stereo Investigator computes a shape factor that can be displayed by clicking **Display>Display Settings>Accessories>Contour Measurements** and scrolling to the right of the **Contour Measurements** window.

Merz

This probe is used to estimate both the ratio of length (perimeter) to (cross-sectional) area and surface area to volume. It uses the curvilinear test system devised by Merz (1967) known as the Merz Coherent Test System. This is a global probe.

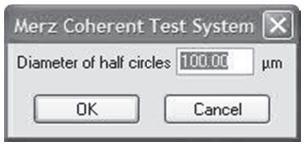
A grid of semicircles laid out in a square lattice is drawn over the material to be examined. Markers are placed wherever the test lines cross the boundaries of the objects of interest.

Prerequisites

It is assumed that if individual objects are being measured, if they are not isotropically shaped, there must be a number of them that are isotropically oriented, or observed in several isotropic sections.

To perform a Merz probe run

1. Click **Probes>Merz**. Stereo Investigator displays the **Merz Coherent Test System** dialog box.



2. Specify the diameter of the semicircle grid elements. This parameter controls both the distance between the grid of half circles which are drawn on the screen and their diameter. As the value of this parameter is made smaller, more intersections can be expected, and the more accurate the estimate will be. You may experiment to determine an appropriate grid size so that a statistically significant number of intersections are achieved.
3. Select a marker.
4. Mark each intersection of a particle's outline with the test grid.
5. Move to as many fields of view as necessary, using **Meander Scan** or any other technique for moving from one field of view to another.
6. When complete, click **Probes>Merz**.
7. Click **Probes>Display Probe Run List** to display the **Previous Stereological Runs** dialog box.
8. Select one or more of the Merz entries, using the left mouse button with the SHIFT or CTRL keys.
9. Click **View Results**.

The estimated ratio of objects' perimeters to their area and their surface area to their volume (surface density) are displayed along with the total number of each type of marker and the grid size.

References

- Weibel, E.R. (1979) Stereological Methods. Vol. 1. Practical Methods for Biological Morphometry. Academic Press, London.

Weibel

This probe is used to estimate the ratio of length to cross-sectional area and surface area to volume. It uses a linear test system devised by Weibel. This is a global probe.

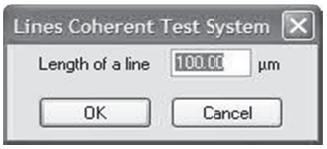
The probe displays a set of short lines laid out in a lattice over the material to be examined. Markers are placed wherever the test lines cross the boundaries of the surface.

Prerequisites

IUR sections are required, or isotropically shaped objects. It is assumed that if objects are not isotropically shaped, there must be a number of them that are isotropically oriented, or observed in several isotropic sections.

To perform a Weibel probe run

1. Click **Probes>Weibel**. Stereo Investigator displays the **Lines Coherent Test System** dialog box.



2. Specify the length of the linear grid elements. This parameter determines the length of the test lines. It also determines the distance from the end of each line to the beginning of its nearest neighbors. You may experiment to determine an appropriate grid size so that a statistically significant number of intersections are achieved.
3. Select a marker.
4. Mark each intersection of an object's boundary with the test grid.
5. Move to as many fields of view as necessary, using **Meander Scan** or any other technique for moving from one field of view to another.
6. Click **Probes>Weibel**.
7. Click **Probes>Display Probe Run List** to display the **Previous Stereological Runs** dialog box.
8. Select one or more of the **Weibel** entries, using the left mouse button with the SHIFT or CTRL keys. Click **View Results.**

The test line length per test area and surface density estimate associated with each object are displayed along with the averages for the group.

References

- Weibel, E.R., et al. (1966) Practical Stereological Methods for Morphometric Cytology. J. Cell Biology 30(1): 23-38.

Discrete Vertical Rotator

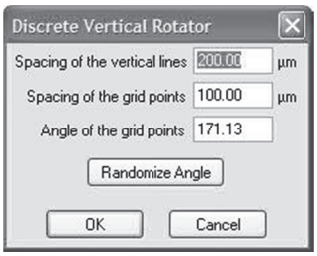
The **Discrete Vertical Rotator** is used to estimate volume of small particles in single extremely thin sections (specifically EM samples) obtained through unbiased sampling schemes. In the paper by Miranov and Miranov (1998) the estimator is described using centrioles as the sampling unit and vertical axis for

the cells being sampled, as the centrioles are easily recognized and are of uniform size between cells. Using the centrioles as a vertical axis, the distance of test points from this axis is used to estimate the volume of other particles in the cell.

Test systems in this probe consist of a grid of lines parallel to the vertical axis, and a grid of points. Points falling on each structure of interest are placed into distance classes based on the distance from the vertical axis. The number of points in each distance class is used to generate a volume estimate.

To perform a Discrete Vertical Rotator probe run

1. Click **Probes>Discrete Vertical Rotator.** Stereo Investigator displays the **Discrete Vertical Rotator** dialog box.



2. Enter the spacing for the vertical lines, spacing for grid points, and optionally, the angle of the grid points. To randomize the angle, click **Randomize Angle**.
3. Click **OK**. A message in the status bar instructs you to click on the centriole of the cell.
4. After clicking on the centriole (or other vertical point) a grid of lines and points is displayed. The vertical lines are parallel to the vertical axis and represent the distance classes into which the selected points are sorted.
5. Right-click and choose **Adjust Vertical Axis**. A large arrow with a T-square at the end is displayed. Adjust the vertical axis so that it is parallel to the alignment of the centrioles. Use the mouse to drag the head of the arrow until it is in alignment with the desired vertical axis.
6. Right-click and select **Accept** to set the new vertical axis.
7. Click on all of the points that fall within the profiles of particles of interest in the field of view. You do not need to select a marker, as tick marks are placed on the grid points. The lines are for reference only, and do not need to be marked.

The shape and size of the tick marks can be changed in the **Options>Stereology Preferences>Colors and Tick Marks** dialog box.

8. Once all points are marked within the particles of interest, right-click and select **Exit Discrete Vertical Rotator**, or click **Probes>Discrete Vertical Rotator**.
9. Click **Probes>Display Probe Run List** to display the **Previous Stereological Runs** dialog box.
10. Select one or more of the **Discrete Vertical Rotator** entries, using the left mouse button with the SHIFT or CTRL keys. Click **View Results.**

Results listed for the **Discrete Vertical Rotator** include the number of points in each distance class (called numbered Bands in the results), and the total estimated volume.

References

- Mironov, A.A. Jr and Miranov A.A. (1998). Estimation of subcellular organelle volume from ultrathin sections through centrioles with a discretized version of the vertical rotator. J. Microsc. 192 (1): 29-36.

Combined Slope Intercept

You can use the **Combined Slope Intercept** probe to estimate profile area and profile boundary length. In using this probe, intersections with linear features (boundaries) are marked on the lines of a grid, while the profile area of the region of interest is estimated by marking the number of grid vertices that fall within the region.

Prerequisites and Parameters

Prerequisites

Thin sections are preferred for this probe. Any orientation of section can be used. To estimate boundary length using vertical sections, use the L-cycloid or IUR Planes probe. To estimate boundary length using preferential sections, thick sections are required, and you can use the Space Balls or Isotropic Virtual Planes probe.

Sampling Parameters

The combined slope intercept probe consists of a grid with the same line separation in the X and Y axes. The separation of the grid lines is the only parameter you are required to set. You can also set the angle of the grid lines, or use the Randomize Angle button to select a random angle. However, if you are using IUR sections, randomizing the grid angle is not necessary.

To perform a Combined Slope Intercept probe run

1. Click **Probes>Combined Slope Intercept**. Stereo Investigator displays the **Combined Slope Intercept** dialog box.
2. Specify the separation between the linear grid elements. You may experiment to determine an appropriate grid size so that a statistically significant number of intersections are achieved.
3. It is not necessary to select a marker. Once a marking method is selected, tick marks are placed for each click of the mouse.

   The shape and size of the tick marks can be changed in the Options>Stereology Preferences>Colors and Tick Marks dialog box.

4. Right-click and select **Mark Line**.
5. Mark each intersection of an object's boundary with the test grid.
6. Right-click and select **Mark Vertices**.
7. Mark each grid intersection that falls within the profile of the region of interest.
8. Move to as many fields of view as necessary, using Meander Scan or any other technique for moving from one field of view to another.
9. Click **Probes>Combined Slope Intercept** or right-click and **select Exit CSI** to end the probe.

10. Click P**robes>Display Probe Run List**.
11. Select one or more of the **Combined Slope Intercept** entries to see results.

Results

Stereo Investigator presents the CSI under the heading **General Results**. Each run of the probe is listed in a separate line. The table headings are as follows:

- **Grid Size:** The separation between grid lines.
- **Intercepts:** The number of intercepts (line markings) in that run of the probe.
- **Profile length:** An estimate of the total profile (boundary) length based on the number of intercepts selected.
- **Vertices**: The number of vertices marked in that run of the probe.
- **Profile area:** An estimate of the total profile area based on the number of intercepts selected.

References

There is not a specific reference for the Combined Slope Intercept probe; it is based on the principles described in the following:

- Miles, R.E. and Pamela Davy (1976). Precise and general conditions for the validity of a comprehensive set of stereological fundamental formulae. J. Microsc. 107(3):211-226.

Point Sampled Intercept

The **Point Sampled Intercept** is used to estimate the volume of cells or other small objects in isotropic or vertically sectioned tissue. It uses a randomly placed array of uniformly spaced lines and points to measure the linear intercept across each object of interest. The points are represented by vertices with shorter lines that are spaced uniformly along the longer lines. A particle is sampled if one of these vertices falls within its profile. The linear intercept is the distance from one boundary to another along the randomly placed line that intersects the object at a specific point (vertex) on the line. The intercepts can fall anywhere in the profile of the object and do not need to be at the center or at any identifying point in the particle. This length is used to estimate a volume of the sampled objects.

The Point Sampled Intercept is more likely to sample a larger cell than a smaller cell, so the results are Volume Weighted. That is, the probability of hitting a particle with the probe is proportional to the volume of the particle.

Prerequisites and Parameters

Prerequisites

The **Point Sampled Intercept** requires an isotropic intercept between the sampling line and the particles being sampled. That is, the direction of the intercept through the sampling point must be isotropic in 3D space with respect to the particles. There are three ways of fulfilling this condition:

1. The particles themselves are isotropic in the tissue (rare, and can be difficult to prove).
2. The sections are isotropic.

3. The sections are vertical—all parallel to a single, recognizable vertical axis. The sections must be isotropic around this axis.

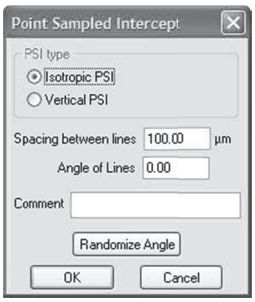
Therefore, unless the isotropy of the particles can be demonstrated, the **Point Sampled Intercept** requires thin sections that are isotropic sections or vertical sections.

Parameters

The **Point Slope Intercept** only requires choosing Isotropic or Vertical orientation, and designating the space between the lines and the angle of the lines. In addition, a comment field allows for the sampling of more than one kind of cell in a single run of the probe.

To perform a Point Sampled Intercept probe run

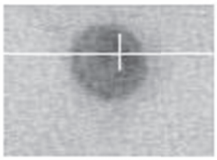
1. Click **Probes>Point Sampled Intercept**. Stereo Investigator displays the **Point Sampled Intercept** dialog box.



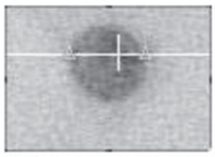
2. Choose a PSI type and then specify the spacing between the lines. You may experiment to determine an appropriate line spacing so that approximately 50-100 intercepts are sampled per specimen.

It is not necessary to select a marker. Once a vertex is selected, you use the mouse to indicate the points at the boundaries of the particle and tick marks are placed.
You can change the size and shape of the tick marks with **Options>Stereology Preferences>Colors and Tick Marks**.

3. Click on a point that falls within the profile of a particle to be sampled.
4. Once the vertex is selected, all other lines and vertices are temporarily hidden, and this display shows only the line and vertex over the particle.



5. Left-click to mark the intersections of the line and the particle boundaries. Tick marks are displayed at the selected intersections.



To delete an erroneously placed point, right-click over the tick mark and select Delete.

6. Once all intercepts for that particle have been marked, right-click and select **Finish Current PSI**.

Once a vertex has been selected, it appears with a tick mark over it so that you will not select it again.

7. Click on all vertices in the current field of view that fall within particle profiles, repeating steps 3-6 for each particle. If more than one vertex falls within a given cell, both points should be marked.
8. Move to as many fields of view as necessary, using **Meander Scan** or any other technique for moving from one field of view to another.
9. To end the probe run, click **Probes>Point Slope Intercept** or right-click and select **Exit PSI**.
10. Click **Probes>Display Probe Run List** to view the results.

Results

The PSI results are presented under **General Results**. The row headings are as follows:

- **Points Sampled**: The number of points sampled (vertices) in that run of the probe.
- **Mean Volume**: The mean volume estimate of all particles sampled in that run of the probe.
- **CE**: The coefficient of error.
- **CV** : The coefficient of variance.
- **pi/3*SD**: This is the basis of the variance calculation, the value of Pi/3 times the standard deviation of the intercept length.
- **Variance**: Calculated by multiplying the above value by the sum of the intercept lengths to the third power.

References

There is not a specific reference for the **Combined Slope Intercept** probe; it is based on the principles described in the following:

- Miles, R.E. and Pamela Davy (1976). Precise and general conditions for the validity of a comprehensive set of stereological fundamental formulae. J. Microsc. 107(3):211-226.

Cycloids for Sv

This probe is used to estimate the surface area per unit volume. This ratio of surface area to volume is sometimes referred to as the surface density.

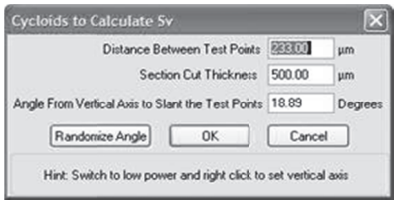
Stereo Investigator displays sets of short test curves (cycloids) and test points in a grid pattern over the material to be examined. You then place markers wherever these cycloids, or test points, cross the boundaries of the surface whose area you want to estimate.

Prerequisites

The Cycloids for Sv probe requires vertical sections with a random rotation around the vertical axis, random sampling with sections, and an identifiable vertical axis.

To perform a Cycloids for Sv probe run

1. Click **Probes>Cycloids for Sv**. Stereo Investigator displays the **Cycloids to Calculate Sv** dialog box.



2. Specify the distance between the test points and their angle relative to the vertical axis. You may experiment to determine an appropriate grid size so that a statistically significant number of intersections are achieved.
3. Type an angle for the vertical axis, or click **Randomize Angle**. You can also change the angle when the probe is visible. Right-click and select **Define Vertical Axis**. Use the cursor to grab the head of the arrow that is displayed and move it so the long axis is aligned with the vertical axis of the tissue. Right-click and select **Accept** when finished defining the vertical axis.
4. Use the left mouse button to click on all the test points (angle marks) inside the region of interest, and also wherever the cycloid curves cross the boundary of the region of interest.
5. Move to as many fields of view as necessary, using Meander Scan or any other technique for moving from one field of view to another.
6. To end the probe, click **Probes>Cycloids for Sv** again or right-click and select **Exit Cycloid**.
7. Click **Probes>Display Probe Run List** to results.

Results

The results displayed are the estimated surface density (Sv), the ratio of test curve points to test curve length (p/l), the number of intersections and the number of test points.

References

- Baddeley, A., H. Gundersen, L. Cruz-Orive (1986) Estimation of surface area from vertical sections. J. Microc. 142 259-276.
- Vesterby, A., J. Kragstrup, H. Gundersen,F. Melsen, (1987) Unbiased Stereological Estimation of Surface Density in Bone Using Vertical Sections, Appendix: The Cycloid Test System. Bone 8 13-17.

Cycloids for Lv

This probe is used to estimate the ratio of total line length to unit volume.

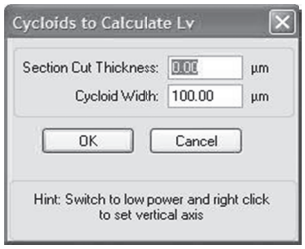
Stereo Investigator displays sets of short test curves (cycloids) in a grid pattern over the material to be examined. Markers are placed where these cycloids cross the projected lineal features of interest.

Prerequisites

The Cycloids for Lv probe requires VUR (**V**ertical, **U**niform, **R**andom) slices of constant thickness with a random rotation around an identifiable vertical axis. The lineal features to be sampled must have a very small cross-sectional area.

To perform a Cycloids for Lv probe run

1. Click **Probes>Cycloids for Lv**. Stereo Investigator displays the **Cycloids to Calculate Lv** dialog box.



2. Specify the section thickness and cycloid width. Stereo Investigator overlays a test grid of cycloids and angles on the image.
3. Right-click and choose **Define Vertical Axis**. Use the cursor to grab the head of the arrow that is displayed and move it so the long axis is aligned with the vertical axis of the tissue. Right-click and select **Accept** when finished defining the vertical axis.
4. Use the left mouse button to mark wherever the cycloid curves cross the lineal features of interest. You do not need to select a marker, as tick marks are used to indicate the location of all marked intersections. Mark intersections in all focal planes of the tissue. Mark the angles wherever they fall within the region of interest.

   You can move from one field of view to the next using either the arrow buttons at the bottom right corner of the Stereo Investigator window, or by using the **Meander Scan** option on the **Move** menu.

5. When done marking all intersections, click **Probes>Cycloids for Lv** again, or choose **Exit Cycloid** from the right-click menu.

Results

Results include general results on cycloid length and width, test points per cycloid, area, length density, and Lv and Length variance information.

References

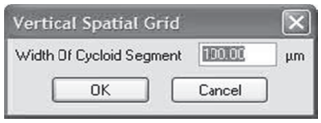
- Baddeley, A., H. Gundersen, L. Cruz-Orive (1986) Estimation of surface area from vertical sections. J. Microc. 142 259-276.
- Vesterby, A., J. Kragstrup, H. Gundersen,F. Melsen, (1987) Unbiased Stereological Estimation of Surface Density in Bone Using Vertical Sections, Appendix: The Cycloid Test System. Bone 8 13-17.

Vertical Spatial Grid

The **Vertical Spatial Grid** is used to estimate the volume and surface area of objects contained within a vertical uniform random stack of parallel sections, such as a confocal image stack. Within each section, an array of parallel cycloid segments and grid points are used to estimate the surface area.

To perform a Vertical Spatial Grid probe run

1. Click **Probes>Discrete Vertical Rotator**. Stereo Investigator displays the **Discrete Vertical Rotator** dialog box.



2. Enter the spacing for the cycloid segments, and click **OK**.
4. Right-click and choose **Adjust Vertical Axis**. Adjust the vertical axis so that it is parallel to the alignment of the vertical axis. Right-click and select **Accept** to set the vertical axis.
5. Click on all of the points where the surface of the object of interest intersects the cycloid array. You do not need to select a marker, as tick marks are placed on the cycloids.
6. To move to the next focal plane, right-click and select **Move Up One Plane** or **Move Down One Plane**.
7. To mark intersections within the region of interest (for estimating area), right-click and select **Mark Grid Points**. An array of points is displayed. Mark all grid points that fall within the profiles of objects or areas of interest.
8. Once all points are marked within the particles of interest, right-click and choose **Exit Vertical Spatial Grid**, or click **Probes>Vertical Spatial Grid**.

Results

Results listed for the Vertical Spatial Grid include the number cycloid and test grid intersections, the estimated volume, and estimated surface area.

Reference

- Cruz-Orive, L.M. and C.V. Howard (1995) Estimation of individual feature surface area with the vertical spatial grid. J. Microsc. 178 (2); 146-151.

Surface Weighted Star Volume

The Surface Weighted Star Volume probe provides as estimate of tissue volume. This estimator is most useful in tissue such as lung or bone tissue, in which the volume of the air spaces (alveoli or marrow space) is to be estimated.

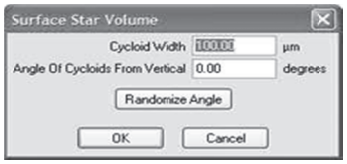
Surface-weighted star volume is the average of the star volumes calculated from points on the phase interface (surface of the phase of interest). At each sampled point, a ray is drawn in an isotropic direction from the surface intersection and the location where the ray intersects the next interface of the phase is marked. From these linear values, a volume is estimated.

Prerequisites

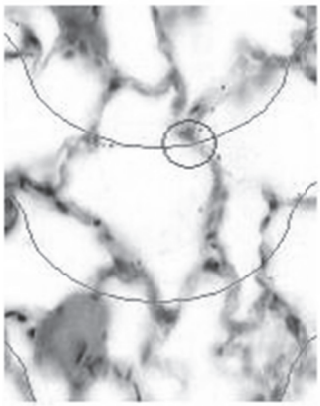
This probe requires vertical sections with random rotation around an identifiable vertical axis and random sampling within sections.

To perform a Surface-weighted Star Volume probe run

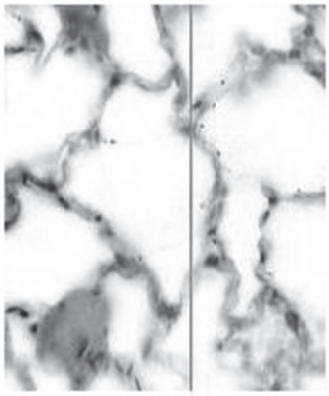
1. Click **Probes>Surface Weighted Star Volume**. Stereo Investigator 8.0 displays the **Surface Weighted Star Volume** dialog box.



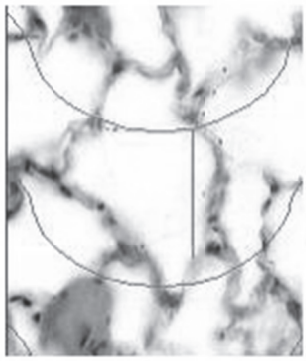
2. The cycloids are an IUR linear test probe that is displayed over the tissue to be sampled. Specify the cycloid width and their angle relative to the vertical axis. You should experiment to determine an appropriate grid size so that a statistically significant number of intersections are achieved. Specify the cycloid width and their angle relative to the vertical axis. Stereo Investigator 8.0 overlays the test grid of cycloids on the slide image. If you need to adjust the Vertical Axis, right-click and select **Define Vertical Axis**. Adjust the vertical axis so that it is parallel to the alignment of the vertical axis. Right-click and select **Accept** to set the vertical axis
3. Left-click to mark the surface of the phase of interest where it intersects with the curves of the cycloids, as seen here.



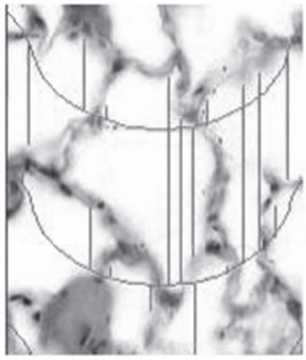
Stereo Investigator hides the cycloids and draws a line parallel to the vertical axis.



Stereo Investigator again displays the cycloids, with a line indicating the marked distance across the phase.



4. Move to as many fields of view using **Meander Scan** or another method, and continue sampling phase interfaces until all have been sampled. In the example here, the result looks like this illustration.



5. Click **Probes>Surface Weighted Star Volume** or right-click and select **Exit Surface Weighted Star Volume.**
6. Click **Probes>Display Probes Run List** to see the results.

Results

The number of intersections, the sum of the cubed lengths of the lines marked, and the total estimated volume appear. If more than three sections have been sampled, the CE is also displayed.

References

- M.G. Reed and C.V. Howard (1997). Surface-weighted star volume: concept and estimation. J. Microsc. 190(3):350-356.

Isotropic Fakir Method

The **Isotropic Fakir Method** uses a linear probe consisting of three mutually orthogonal lines to estimate surface area in thick sections (slices). This probe can be used on sections cut in any orientation, as the probe lines themselves are isotropic in orientation. The number of intersections between the triplets of probe lines and the surface of the sampled object are counted, and surface area estimated from these counts.

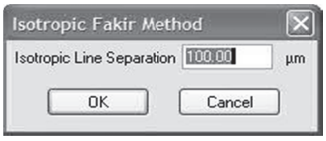
Prerequisites

This probe requires thick tissue sections (slabs) in which a number of adjacent optical sections can be sampled. Tissue can be sectioned in any orientation.

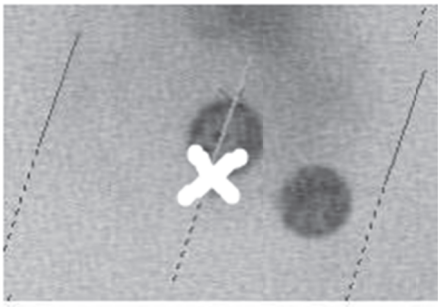
To perform an Isotropic Fakir Method probe run

If you are sampling serial sections, start the **Serial Section Manager** first.

1. Click **Probes>Isotropic Fakir Method**. Stereo Investigator displays the **Isotropic Fakir Method** dialog box.



2. Enter the line separation. **Isotropic Lines Separation** refers to the three sets of mutually orthogonal test lines. You should experiment to determine an appropriate line spacing so that a statistically significant number of intersections are achieved.
3. Click **OK**. Stereo Investigator displays an array of parallel line fragments. The lines are displayed as solid to represent the part of the line above the current plane, dashed to show the part below the current plane.
4. Select a Marker.
5. Place a marker wherever a line intersects the boundary of the particles or sampled region, where that boundary appears in the most clear focus, as seen in this example.



6. Focus up and down until all particle boundary intersections in the current field of view are marked.
7. Once all intersections in the current field have been marked, right-click, select Line Set and switch to the second line orientation.
8. Mark all boundary intersections with the new orientation of lines, then repeat for Line Set 3.
9. Once all line sets have been sampled, move to the next field of interest and sample all of the line sets in the new field. Repeat until all fields of interest have been sampled.
10. Click **Probes>Isotropic Fakir Method** again or right click and select **Exit Isotropic Fakir**.

Results

The results of the Isotropic Fakir Method probe list the line separation, the number of intersections in each line set, and the total estimated surface area in microns squared.

References

- Kubínová, L. and J. Janacek (998). Estimating surface area by the isotropic fakir method from thick slices cut in an arbitrary direction. J. Microsc. 191(2):201-211.
- Cruz-Orive (1997). Stereology of single objects. J. Microsc. 186:93-107.

Nucleator

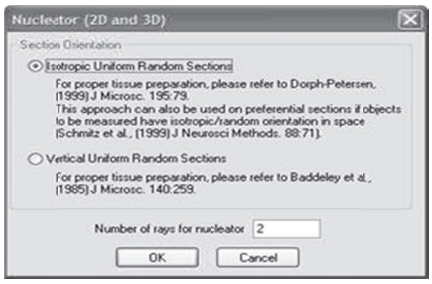
The Nucleator probe can be used to estimate the mean cross-sectional area and volume in IUR sections of objects such as cells. The Nucleator is a local probe, usually used within the context of an Optical Fractionator or another systematic uniform random sampling scheme to identify the cells to be sampled. If you have a small population, all cells can be sampled with the Nucleator.

By identifying where a set of rays emanating from the chosen point associated with each object cross the object's boundaries the mean area and volume of the sampled objects can be estimated. It is generally recommended that the associated point for each object should be placed at a consistent location within the object, such as the nucleus or nucleolus, although this is not necessarily required. All Nucleator rays do not necessarily need to intersect the object. A ray might intersect an object multiple times, depending on the shape of the object and the location of the Nucleator point associated with the object.

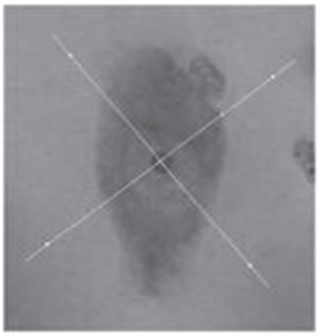
To perform a Nucleator probe run

1. Use the Optical Fractionator or another systematic uniform random sampling scheme to identify cells to be sampled.

2. Once the first cell has been identified, but before clicking on it, click **Probes>Nucleator**. Stereo Investigator displays the **Nucleator Parameters** dialog box.



3. Select **Isotropic Uniform Random Sections** or **Vertical Uniform Random Sections** based on the type of sections used, indicate the number of rays to be used, and then click **OK**.
4. Select a marker to identify a particle, such as a cell.
5. Click on the unique point within the particle or cell (the nucleolus is typically used for cells). The rays are shown extending out from the central point.



6. Click on the location where each of these lines intersects the boundary of the particle. If a line crosses the boundary multiple times, click at each intersection. Stereo Investigator places tick marks at each boundary.

To delete incorrect tick mark placements, right-click over the tick mark and choose **Delete**.

7. When finished marking the boundaries of the current particle right-click and choose **Finish Current Nucleator**.
8. Click on the next particle in the counting frame to be sampled and use the techniques described above to mark where the lines of the Nucleator rays cross its boundaries.
9. When all particles in a given sampling frame have been sampled and the last Nucleator finished, right-click and choose **Next Scan Site** to move to the next sampling frame and begin Nucleator sampling again.
10. When you are done marking all the particles of interest, right-click and select **Finish Current Nucleator and Exit**, or click **Probes>Nucleator** again.

Results

The **Results** displayed include the volume estimate associated with each object, along with the average volume for the group of objects measured. If the Nucleator is run within the context of an Optical Fractionator, an estimated total number of cells is also displayed.

References

- Gundersen HJG (1998). The Nucleator. J. Microsc. 151(1): 3-21.
- Schmitz C., et al. (1999) No difference between estimated mean nuclear volumes of various types of neurons in the mouse brain obtained on either isotropic uniform random sections or conventional frontal or sagittal sections. J. Neurosci Methods. Apr 1;88(1):71-82.
- Dorph-Petersen KA. (1999) Stereological estimation using vertical sections in a complex tissue. J Microsc. 195(Pt 1):79-86.
- Baddeley AJ, et al., (1986) Estimation of surface area from vertical sections. J Microsc. (142):259-276.

Planar Rotator

The **Planar Rotator** probe can be used to estimate the volume of particles, such as cells, in isotropic and vertical sections. The **Planar Rotator** is a local probe, usually used within the context of an Optical Fractionator or another systematic uniform random sampling scheme to identify the cells to be sampled. If you have a small population, all cells can be sampled with the **Planar Rotator**.

Prerequisites

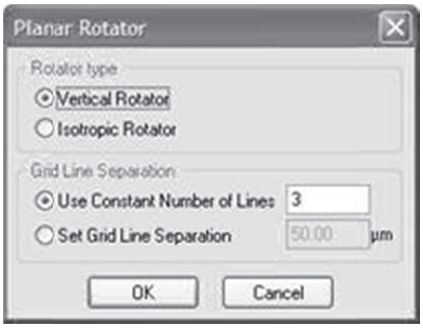
This probe requires:

- Sections cut with uniform random position in the reference space.
- Sections have either isotropic or vertical orientation.
- Sampling must be systematic within and between sections.
- A unique reference point is associated with each particle.

The first three requirements essentially ensure that estimates for particles that are not isotropic in shape, orientation or distribution will not be biased by these factors. Use of a unique reference point for each particle (for instance the nucleus of a cell) helps ensure that measurements are made at random depth along the Z-axis. For instance, measuring each cell at a depth where its largest cross-section is visible might bias the resulting volume estimates. It is usually assumed that a number of particles will be sampled and measured. The resulting average represents mean particle volume.

To perform a Planar Rotator probe run

1. Click **Probes>Planar Rotator**. Stereo Investigator displays the **Rotator Parameters** dialog box.

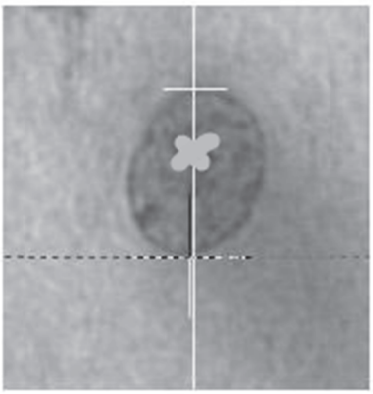


2. Specify **Vertical Rotator** or **Isotropic Rotator** based on the type of sections being used.
3. Select **Use Constant Number of Lines** or **Set Grid Line Separation**. If you choose the later, enter the desired grid line separation, and then click **OK**.
4. Select a marker with which to mark objects to be measured.
5. Click on an object to be measured. It is recommended that you always pick the same relative point for each object, for instance the nucleolus of cells. A single line is drawn through the reference point of the object.

Vertical Sections: If you are using vertical sections, the line should be parallel to the vertical axis of the tissue. To change the orientation of the line, position the cursor over one of the small squares on the vertical line. An icon depicting a human hand will appear. Grab the vertical line by clicking on the left mouse button and align it with the vertical axis for this section. If you are using isotropic sections you can align this vertical line along the major axis of the particle to be measured; this will usually help improve the variance of the estimate.

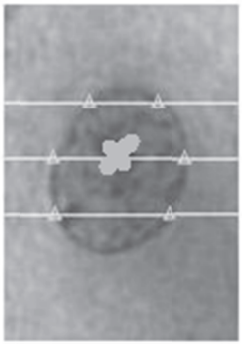
Isotropic Sections: For isotropic sections, align this vertical line along the major axis of the particle to be measured. This will usually help improve the variance of the estimate.

6. Click on the line at each point that it crosses the boundary of the object. If a line crosses multiple boundaries, place a marker at each point. This figure show the vertical line with the top intersection marked and the bottom intersection about to be marked.



(The dashed line indicates where the intersection mark will be placed.)
A series of horizontal lines will be drawn, equidistant from each other. A vertical line will also be drawn through the object you are measuring.

7. Click at the point(s) each horizontal line crosses a boundary of the object being measured. At each point, a tick mark is placed (the triangles in this illustration). If a line crosses multiple boundaries, place a tick mark at each point. This is illustrated here.



To delete incorrect tick mark placements, right-click over the tick mark and choose Delete.

8. When done marking the boundaries of the current object, right-click and choose **Finish Current Planar Rotator**.
9. Click on the next object to be measured and use the same techniques listed above to mark where the lines of the Planar Rotator overlay cross the object's boundaries.
10. When you are done marking all the objects of interest, right-click and select **Finish Current Planar Rotator and Exit**, or click **Probes>Planar Rotator** again.
11. Click **Probes>Display Probe Run List** to display the results.

Results

In the Planar Rotator Sampling Results dialog box, the volume estimate associated with each object is displayed, along with the average volume for the group of objects measured. If the Planar Rotator is run within the context of an Optical Fractionator, an estimated total number of cells is also displayed.

Optical Rotator

The **Optical Rotator** is used to estimate the volume and surface area of particles, such as cells, in thick, transparent slabs. The optical rotator may be used with either isotropic or vertical slabs, but surface area may be estimated only from isotropic slabs.

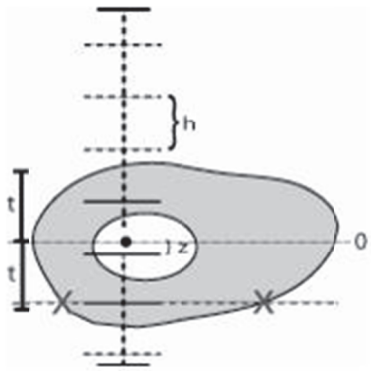
Note that the term "slab" is used to refer to what is more commonly known as a "section". This terminology follows that used by the authors who first published this technique. A "slice" refers to an optical slice, a 2D plane through a tissue slab, the slab being a 3D structure with thickness.

The optical rotator collects information in 3D and samples focal planes in a virtual slice through the central region of the cell, avoiding the complications of trying to determine boundaries at extremes of the cell. Probes such as the Cavalieri estimator can also be used to determine volume of individual cells, but there is a problem of determining cell boundaries at the top and bottom of the cell, where the boundaries are generally blurred and overprojection can be a problem.

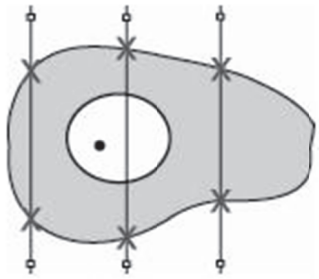
A systematic uniform random sampling scheme such as an optical fractionator should be used to identify the cells to be sampled. After identifying a particle for sampling, **Stereo Investigator** displays an overlay composed of a set of parallel lines through a systematic series of focal planes. The lines are placed

alternately parallel or perpendicular to the grid axis at each focal plane. By identifying where these lines intersect a particle's boundaries (in the X's in the figures in this topic) at different focal depths the surface area and volume of particles can estimated.

XY (top) view of sampling lines in a single focal plane.



XZ (side) view of optical slice (2t) through a cell and enclosed focal planes illustrates the layout of the Optical Rotator in a "side view", or XZ projection.



Parameters to be entered in the **Optical Rotator** setup by the user are *italicized* in this description. The unique point of the cell (e.g. nucleolus) is marked, and its Z-depth designated as the origin (0). If there is no nucleolus, or multiple nuclei, a single unique point must be designated for each cell. This can be the top of the nucleus (the point at which the nucleus first comes into focus), the first nucleus encountered, or some other unique point.

The *optical slice thickness* is 2t, with 0 as the center of the slice. This parameter can be different for every cell, and should be placed so that the extremes of the cell are excluded. If the cell has a shape different from a sphere, use the smallest diameter of the cell to determine the thickness of the 2t slice. In most cases it is sufficient to use about half of the smallest diameter of the cell as the 2t value. If the cells are nearly spherical and approximately the same size, the same value of 2t can be used for all cells, although this is generally not the case. For purposes of clarity, the example in the first figure above shows a value for 2t that is too large, as it is nearly the thickness of the cell. 2t should more closely approximate the smallest radius of the cell.

The *focal plane separation* (h) is determined by the user to allow for the desired number of focal planes within the optical slice of the tissue. In this illustration, 3 focal planes fall within the slice. The placement of the focal planes is determined by a starting point a distance z from the origin, with z being a random number between 0 and h. Focal planes that do not fall within the optical slice are shown as dashed horizontal lines. The vertical dashed line indicates the overall thickness of the section (called the slab in the original papers, see References below), with short horizontal bars at each end showing the extent of the

tissue section. The placement of a red x demonstrates where the user will mark the boundary of the cell in a single focal plane. The first figure shows the *number of grid lines* as three, with the *initial grid orientation* as vertical.

A total of 8-10 intersections between the cell boundary and the grid in all the optical planes is usually sufficient if the cell is generally spherical or ellipsoid in shape. (This includes all intersections in all focal planes.) If a cell has many involutions or a complex profile, more intersections may need to be counted. The precision of the estimates is dramatically improved when the number of focal planes sampled is increased from one to two planes. An increase to four planes shows some further increase in precision, but also doubles the amount of work. Stereo Investigator's Optical Rotator allows for automatic placement of many sampling planes and easy marking of boundary locations.

The *optical rotator* is a local probe. As with all local probes, one of the key steps is to obtain a systematic, uniform random sample of particles. This is easily accomplished by using the fractionator approach. A powerful feature of **Stereo Investigator** permits the use of the optical rotator for estimating the volumes of cells that are counted while using the optical fractionator. Using this probe with an optical fractionator or other fractionator uniform sampling technique provides number weighted results. Without using such a systematic random sampling procedure, one would obtain volume weighted results, which would be undesirable.

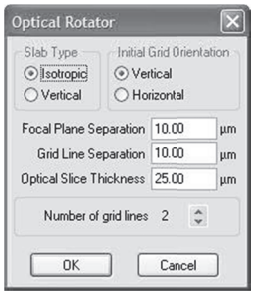
Prerequisites

- A unique reference point is associated with each particle or cell.
- The material has been sectioned isotropically or vertically, or contains particles of isotropic orientation (this assumption must be tested).
- Thick, transparent sections.
- The boundary of the particle to be measured can be seen clearly at different focal depths.

To perform an Optical Rotator probe run

Use the Optical Fractionator or another systematic uniform random sampling scheme to identify cells to be sampled.

1. Once the first cell has been identified, but before clicking on it, click **Probes>Optical Rotator**. Stereo Investigator displays the **Optical Rotator Parameters** dialog box.



2. Select a **Slab Type** and **Initial Grid Orientation**.
3. Enter the **Focal Plane Separation, Grid Line Separation,** and **Optical Slice Thickness**, which should be about half the thickness of the cell.

4. Select the **Number of grid lines** to allow for the desired number of grid lines to fall within the boundaries of a typical cell.
5. Select a marker to identify a particle, such as a cell.
6. Click on the unique point within the particle. The focus position is moved to the first focal plane and a grid consisting of a set of parallel lines are drawn. **Do Not Change The Z-position.**
7. Click on the location where each of these lines intersects the in-focus boundary of the particle. If a line crosses the boundary multiple times, place a marker at each intersection.
    - All lines do not necessarily need to intersect the particle. A line might intersect a particle multiple times, depending on the shape of the particle and the location of the point associated with the particle. Each intersection should be marked.
    - Only mark intersections if the selected focal plane lies well within the volume of the particle to be marked, i.e. avoid sampling near the extreme top and bottom of cells. Stereo Investigator automatically selects valid focal planes if you select the appropriate values for Focal Plane Separation and Optical Slice Thickness.
    - If the lines are too short relative to the cell diameter, you can grab the handles (white boxes) on the lines and extend them outwards.
8. When you have completed marking the intersections on the current focal plane, right-click and select **Move to Next Focal Plane**. The focus is changed to the next focal plane, and the grid is displayed at a perpendicular orientation to that in the previous focal plane.
9. Mark all of the intersections in this focal plane. Repeat this step until all of the focal planes in the particle have been completed. If the particle is no longer in focus after moving to a new focal plane, click **Finish Current Optical Rotator** to indicate you have finished marking this particle. All subsequent focal planes are ignored for this particle.
10. When finished marking the boundaries of the current particle in all focal planes, right-click and select **Finish Current Optical Rotator** from the right-click menu.
11. Click on the next particle to be measured and use the same steps listed above to mark where the lines of the Optical Rotator grid cross its boundaries.
12. When all particles in a given sampling frame have been sampled and the last Optical Rotator finished, right-click and select **Next Scan Site** to move to the next sampling frame and begin Optical Rotator sampling again.
13. When you are done marking all the particles of interest, right-click select **Finish Current Optical Rotator and Exit**, or click **Probes>Optical Rotator**.
14. Click **Probes>Display Probe Run List** to see the results.

Results

Results for the Optical Rotator include Average Volume and Surface Area for all particles sampled, as well as an estimated Volume and Surface Area for each individual particle. If the Optical Rotator is run within the context of an Optical Fractionator, an estimated total number of cells is also displayed.

References

- Tandrup, T., H. Gundersen, E. Jensen (1997). The Optical Rotator. J. Microsc. 186:108-120.

- Kieu, K. & E.b. Vedel Jensen (1993). Stereological estimation based on isotropic slices through fixed points. J. Microsc. 170 (1): 45-51.

Surfactor

The **Surfactor** probe can be used to estimate the absolute surface area of particles of arbitrary shape such as cells.

By identifying where rays are emanating from the chosen point that is associated with each particle crosses the particle's boundaries, and by adjusting tangential rays at these points the mean surface area of particles can be estimated.

The arbitrary point for each particle should be consistent from particle to particle, and does not need to lie inside of the particle.

All surfactor rays do not necessarily need to intersect the particle. A ray might intersect a particle multiple times, depending on the shape of the particle and the location of the arbitrary point associated with the particle.

The surfactor is a local probe, and like all local probes, one of the steps is to obtain a systematic, uniform random sample of particles. This is easily accomplished by using either the physical disector or the optical fractionator.

Prerequisites

- Sections have isotropic orientation.
- An arbitrary point is associated with each particle.
- Use of a unique reference point for each particle (for instance the nucleus of a cell) helps ensure that measurements are made at random depth along the Z-axis. For instance, measuring each cell at a depth where its largest cross-section is visible might bias the resulting volume estimates.

To perform a Surfactor probe run

Use the Optical Fractionator or another systematic uniform random sampling scheme to identify cells to be sampled.

1. Once the first cell has been identified, but before clicking on it, click **Probes>Surfactor**. Stereo Investigator displays the **Surfactor** dialog box.

2. Type the number of rays associated with each measurement.
3. Select a marker.
4. Click on a particle to be sampled. The selected number of rays is drawn, equiangular from each other and outward from the arbitrary point.

You can extend the length of the rays by clicking on the small square near the end of a ray and moving the mouse while holding down the left mouse button. The cursor changes to a hand to indicate that the square can be grabbed.

5. Left-click at the point each ray intersects the boundary of the particle being measured. If a ray intersects the boundary multiple times, place a mark at each point. If you inadvertently placed a marker at an incorrect location, you can remove the marker by positioning the cursor over it, right-clicking and selecting **Delete**.
6. Stereo Investigator draws a tangent line perpendicular to the ray crossing the boundary. Use the left mouse button to grab one of the two small squares near the end of this line and rotate it until it lies tangential to the boundary of the particle.
7. When the tangent on the first ray has been correctly set, right-click and select **Mark Next Tangent** to mark the tangents on the remaining rays.
8. When done marking the boundary of the current particle, right-click and select **Finish Current Surfactor**.
9. Click on the next particle to sample and use the same steps listed above.
10. When you are finished sampling particles, right-click and select **Finish Current Surfactor and Exit** or click **Probes>Surfactor**.

Results

The Results display for the **Surfactor** includes the estimated surface area for each particle sampled, as well as an average surface area for all sampled particles. If the **Surfactor** is run within the context of an **Optical Fractionator**, an estimated total number of cells is also displayed.

References

- Jensen E, H Gundersen (1987), Stereological estimation of surface area of arbitrary particles. Acta Stereologia 6/III:25-30

Linear Disector

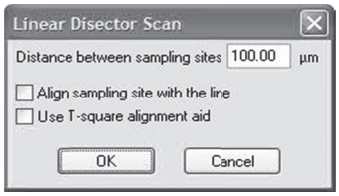
The **Linear Disector** probe combines the **Linear Scan** with a 2D counting frame at each sampling site. It allows a series of evenly spaced sampling sites, laid out in a straight line, to be defined and traversed. This probe can be thought of as a one-dimensional fractionator probe. Often the linear optical disector is used to analyze material that is perpendicular to specific landmarks. The T-square alignment aid is useful for this.

Prerequisites

As with the Linear Optical Disector the distance between sampling sites needs to be specified.

To perform a Linear Disector probe run

1. At low magnification, perform a tracing of the area that you want to scan.
2. Switch to a lens that is suitable for counting cells.
3. Click **Probes>Define Counting Frame** and define the size of the counting frame in the XY plane.
4. Click **Probes>Linear Disector**. Stereo Investigator displays the **Linear Disector** dialog box.



5. Type the desired distance between sampling sites.
6. Choose to align the sampling site with the line and/or to use the T-square alignment aid, and then click **OK**.
6. Stereo Investigator then displays an overview of any existing tracings. Left-click once to set the initial point of the line along which the sampling sites are to be placed.
7. Click again to set the end point of the scan.
8. You are then positioned at the first sampling site.
9. Select a marker for counting cells.
10. Count cells within the selected counting frame by placing markers on the cells to be counted. Any number of marker types can be used for different cell populations.
11. Click the Next Scan button to move to the next scan site.
12. Repeat steps 10-11 until you have examined all of the sample frames in the scan matrix.

Results

Click **Probes>Display Probe Run List**. The results for this probe list the sampling parameters, as well as the number of markers placed at each sampling site. As this is not a published probe, only the raw data is provided, without estimated totals.

Linear Optical Disector

This probe combines the **Linear Scan** and disector probe into a useful surveying tool. The Linear Optical Disector is designed to allow the sampling of sites that are defined by a line (vector) between two locations. Each site will allow the application of an optical disector to allow the counting of cells. You may choose to have the counting frame displayed normally, or you may have it aligned to the orientation of the scan vector.

The **Linear Optical Disector** is often used to analyze material that is perpendicular to specific landmarks. The T-square alignment aid is useful for this.

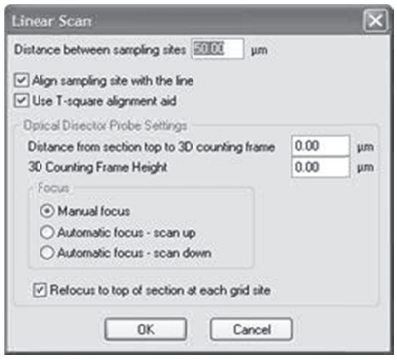
Prerequisites

As with the Linear Disector, the distance between sampling sites needs to be specified. Since an optical disector probe will be implemented at each scan site, parameters such as section thickness, counting frame thickness and guard zone thickness will also need to be determined.

To perform a Linear Optical Disector probe run

1. At low magnification, perform a tracing of the area that you want to scan.
2. Switch to a lens that is suitable for counting cells.

3. Click **Probes>Define Counting Frame** and define the size of the counting frame in the XY plane.
4. Click **Probes>Linear Optical Disector**. Stereo Investigator displays the **Linear Scan** dialog box.



5. Type the desired distance between sampling sites, and then choose to align the sampling site with the line and/or use the T-square alignment aid.
6. Set the **Optical Disector Probe Settings**. Type the distance from the top to the 3D counting frame and the 3D counting frame's height.
7. Choose the focus type, Manual, Automatic focus up, or Automatic focus down.
8. If you want to refocus to the top of each section at each grid site, select this option.
9. Click **OK**. Stereo Investigator displays an overview of any existing tracings. Left-click once to set the initial point of the line along which the sampling sites are to be placed.
10. Click again to set the end point of the scan.
11. You are then positioned at the first sampling site and prompted to focus at the top of the section.
12. Focus at the top of the section and then click **OK**.
13. Select a marker for counting cells.
14. Count cells within the selected counting frame by placing markers on the cells to be counted. Any number of marker types can be used for different cell populations.
15. Click the **Next Scan** button to move to the next scan site.
16. Repeat steps 11–15 until you have examined all of the sample frames in the scan matrix.

Results

Click **Probes>Display Probe Run List** to see results. The results for this probe list the sampling parameters, as well as the number of markers placed at each sampling site. As this is not a published probe, only the raw data is provided, without estimated totals.

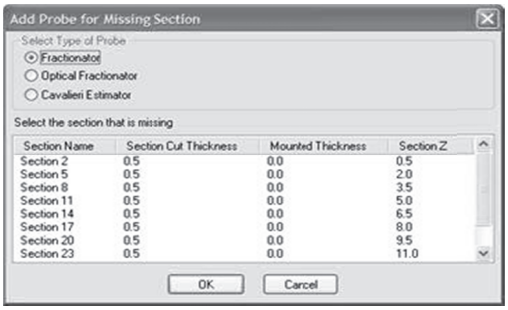
Account for Missing Section

Suppose that for one reason or another a section that you should use for counting is missing. What should you do in this case? Skip the section? Use another section? Make a "guestimate"?

Stereo Investigator includes the **Account for Missing Section** command to help you with a missing section.

To use Account for Missing Sections:

1. Click Probes>Account for missing sections. Stereo Investigator displays the **Add Probe for Missing Section** dialog box.



2. Select the type of probe, Fractionator, Optical Fractionator, or Cavalieri.
3. In the list, select the section that is missing.
4. Click **OK**. Stereo Investigator marks the section as missing. When you look at results, this section is marked as missing.

How Important are Missing Sections?

The important issue here is why the section cannot be used in counting. If the reason is random, then there are stereologically sound methods of using the samples despite the missing section. A dropped section, a section ruined during slicing, a section that did not stain properly are possible random causes. All that matters is that the section is not dismissed for preferential reasons. Such reasons might be that the section had very little of the region of interest on it, or the cells were too crowded to make counting easy. Removing a section for random reasons is similar to applying the fractionator principle. Suppose there are 6 sections. Roll a die and a number from 1 to 6 is chosen at random. Remove that section. Now the remaining 5 sections represent 5/6 of the original. Therefore, an unbiased estimate of the original quantity is the estimate from the 5 sections divided by the sampling fraction which is 5/6.

An important consideration is that although the result is unbiased, the variance has been increased.

If the sections are well spread out it is also possible to substitute a neighboring section. Supposed every 15th section is being used. If it is available, then use the 14th or 16th section as a substitute. Report this substitution in the results. This technique may be more difficult to justify if every other section is being used.

Select Acquired SRS Series

Displays the **Select an Acquired Systematically Random Samples (SRS) Series for Analysis** dialog box.

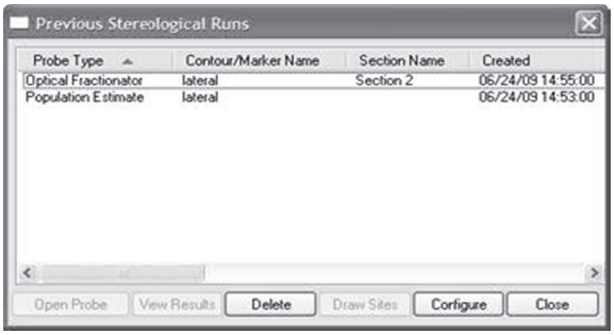
1. Select the SRS series from the list.
2. Select a probe from the drop-down list.
3. Fill in the estimator size, counting frame height and width, and then click **OK.** Stereo Investigator starts a probe run on the SRS series.

Display Probe Run List

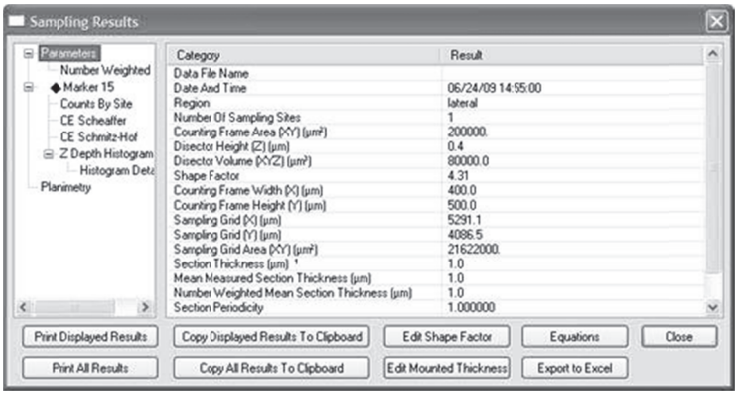
Use this command to view and work with the results of probes. Stereo Investigator displays a list of all the previous stereological runs associated with the current data set. The entries in the **Probe Type, Contour/Marker Name, Section Name,** and **Last Modified** columns of this list can be used to identify each run.

To display results

1. Click **Probes>Display Probes Run List**. Stereo Investigator displays the **Previous Stereological Runs** dialog box.



2. Select a probe or probes from the list. Use the SHIFT key to select consecutive probes, use the CTRL key to select non-consecutive probe runs.
3. Click **View Results**. Stereo Investigator opens the **Sampling Results** dialog box.

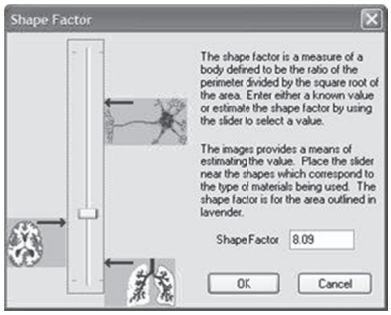


4. The right column contains details related to the heading selected in the left column. Select another heading in the left column to see more details.
5. From here you can use any of the available buttons, explained below.

Sampling Results dialog box buttons

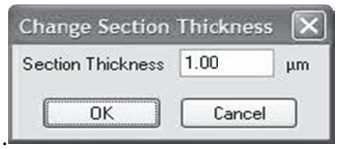
- **Print Displayed Results:** Prints the results displayed.
- **Print All Results:** Prints all the results for the selected probe run.
- **Copy Displayed Results to the Clipboard:** Copies the displayed results to the Windows Clipboard.

- **Copy All Results to the Clipboard:** Copies all the selected probe run results to the Windows Clipboard.
- **Edit Shape Factor:** Displays the **Shape Factor** dialog box.



The CE for the area and volume estimates shown in the Sampling Results dialog box rely on a value known as the Shape Factor. In order to obtain as accurate an estimate of the CE as possible, you should select a **Shape Factor**, which describes the shape of the region whose area or volume is being estimated. This button allows you to change this variable and observe the changes in the resulting statistics.

- **Edit Mounted Thickness:** Displays the **Change Section Thickness** dialog box



Use this if you need to make adjustments to the section thickness.

- **Equations:** Displays an **Equations** dialog box. The equations displayed are related to the probe run.
- **Export to Excel:** Opens Microsoft Excel and exports the selected probe run's data to an Excel Workbook. You must have Microsoft Excel 2003 or later to use this command.

Export to Excel is one way. Any changes you make in the Excel Workbook are not reflected in Stereo Investigator.

- **Close:** Closes the dialog box.

What else can I do with the Previous Stereological Runs dialog box?

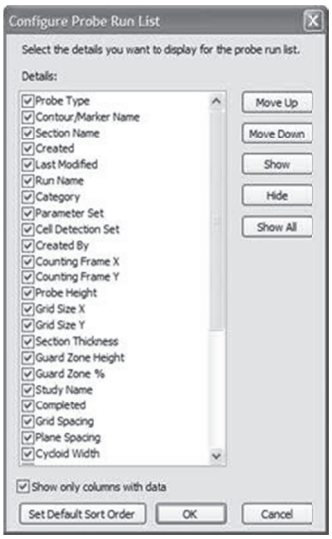
In addition to viewing the results of your probe runs, you can also:

- **Open Probe:** Restarts the probe and lets you revisit each scan site for that probe. This effectively allows a second reviewer to revisit each scan site of any given probe run either to re-run it for themselves, or to visually check the results obtained by a co-worker. This command is also useful to complete work on a probe that had been terminated before all of the sites were visited. In this case, the probe will be restarted at the scan site that was visited last.

If the markers placed for the first run are turned off, a second run can then be executed without being visually biased by the markers placed during the first run.

If a second run is to be made after the synchronization between the tracing and a given section has been lost (for instance if the slide is removed from the microscope), the tracing may need to be realigned with the slide before the original run can be accurately reviewed. The Match function in the Tools menu, or the Rotate Tracing function in the editor can be used to do this.

- **Delete:** Deletes the selected probe run results.
- **Draw Sites:** Use this to view the counting frames used for the probe run.
- **Configure:** Displays the **Configure Probe Run List** dialog,



which you use to add, remove, and configure the details Stereo Investigator displays in the **Sampling Results** dialog box.

- **Close:** Closes the dialog box.

What results does Stereo Investigator display?

Stereo Investigator displays any results for the selected probe. Some of the results are the same for any probe—probe name, contour/marker type, creation time, etc. Other results are specific to a probe. For the results that Stereo Investigator can display for each probe, see the **Results** section of the probe's listing here in the Stereo Investigator Help.

Remember that you can use the **Configure** button in the **Previous Stereological Runs** dialog box to modify and rearrange the details that Stereo Investigator displays.

What can I do with the results?

Once you have your results, you can copy just the displayed results or copy all the results to the Windows Clipboard. From there, you can paste the results into a Word document, Powerpoint presentation, Excel spreadsheet or other document. Remember that if you change any results that you have copied, they are not updated in Stereo Investigator. And if you later make any changes in Stereo Investigator, the changes will not show up in a document unless you copy and paste them again.

If you have Microsoft Excel 2003 or later, you can export directly to an Excel workbook, with the results already formatted and arranged for your use and analysis.

Resample Oversample

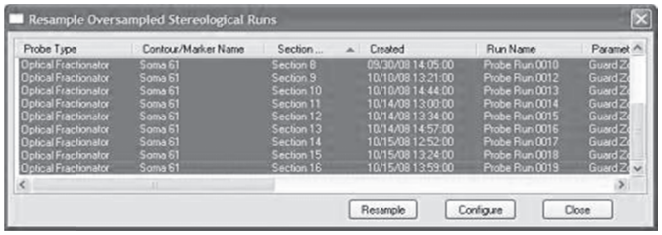
The resample-oversample process is a useful tool to help you understand the precision of the estimate obtained with the Optical Fractionator. When estimating the number of particles in a given region using the Optical Fractionator, you have to decide how much to sample. This decision is affected by the heterogeneity of the particles and the desired precision. You cannot control the spacing of the particles but you can control how much is sampled. The trick is to sample enough to get the desired precision, but not so much that you end up wasting valuable lab time. One way to help gauge the precision of your estimate is to oversample a representative animal from each group as part of your pilot study. Make your disector spacing and section interval two to three times less than you would normally use to sample the region. In other words, take your starting-point parameters (parameters are the disector spacing and section interval) that you have arrived at by choosing parameters that you think will sample all of the salient features of the tissue, and double or triple the amount of sampling. For example, if you think that skipping every tenth section will sample the tissue and result in your desired precision, then decrease the interval to every fifth or every third section for the oversampled animal to increase the number of slides examined. Now you can take this oversampled animal and resample the data. Stereo Investigator will generate a graph that shows the section interval vs. the estimates for those intervals and a graph that shows disector spacing vs. the estimates for those disector spacings. The resample-oversample routine will go through your data and give the estimates not only for the initially oversampled data from your pilot animals but also for cases where the disector spacing or section interval is greater.

Prerequisites

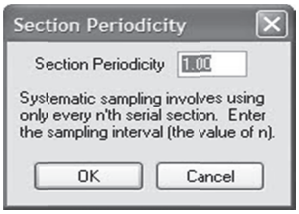
- You will need at least one complete probe run from an oversampled region of interest.
- You must have Microsoft Excel 2003 or later installed to use this command.

To perform a Resample Oversample probe run

1. Click **Probes>Resample Oversample**. Stereo Investigator displays the **Resample Oversampled Stereological Runs** dialog box.



2. Select all the sections for the resampled region. Press SHIFT to select a series of contiguous runs. To select runs not contiguous, press CTRL and then click the runs you want. Click **Resample**. Stereo Investigator displays the **Section Periodicity** dialog box.



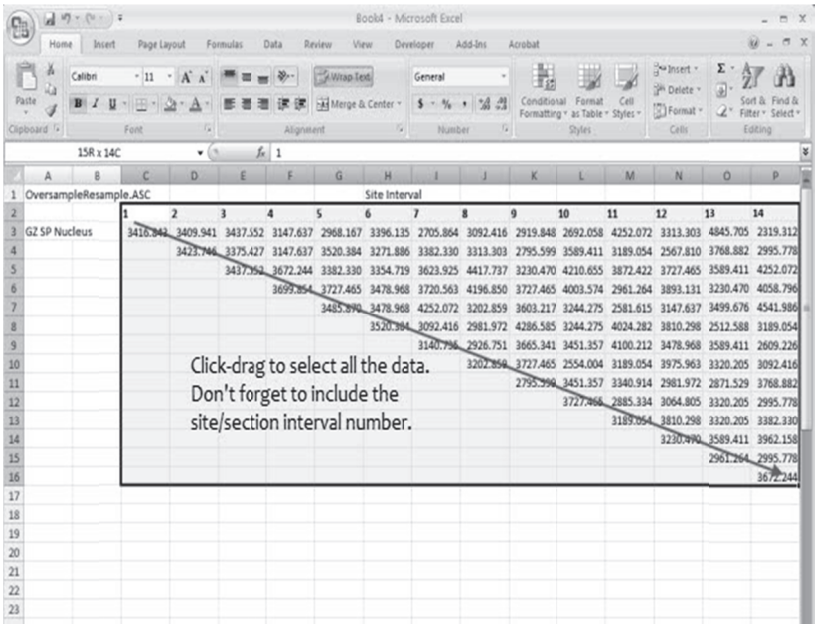
3. If you wish, change the periodicity and click **OK,** or just click **OK**. Stereo Investigator begins the resample oversample process. When complete, Stereo Investigator opens a Microsoft Excel workbook with the results.

Results

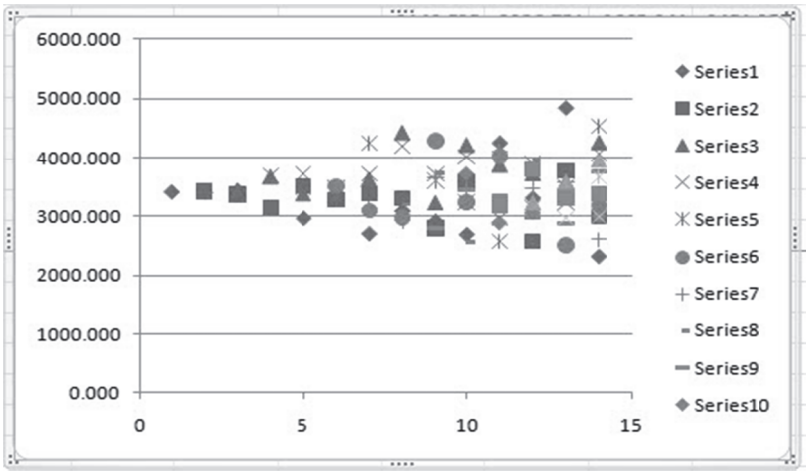
Stereo Investigator opens a Microsoft Excel workbook with your results in the following tabs:

- Mean Estimate by Site: corresponds to the estimated population using mean section thickness.
- Mean Estimate by Section: corresponds to the estimated population in the probe run results.
- Mean-Count by Site.
- Mean Count by Section.
- NW Estimate by Site.
- NW Estimate by Section.

1. Select the **Mean Estimate by Site** tab.
2. Starting at the number 1, click and drag to select all the data from that column to the last column, as shown below:



3. In Microsoft Excel, click **Insert>Scatter** chart, and select the first chart-type shown. Microsoft Excel inserts a scatter chart of your data.



You can also generate graphs for the other tabs. Examine these graphs and find a "sweet spot". According to this representative animal where the parameters are such that you do enough work (have close enough section and disector spacing) so that you get the required precision, but not too much work so that your precision is not unnecessarily tight.

References

- L. Slomianka and M. J. West(2005) Estimators of the Precision of Stereological Estimates: An Example Based on the CA1 Pyramidal Cell Layer of Rats. Neuroscience 136(3), 757–767

Resample Disector

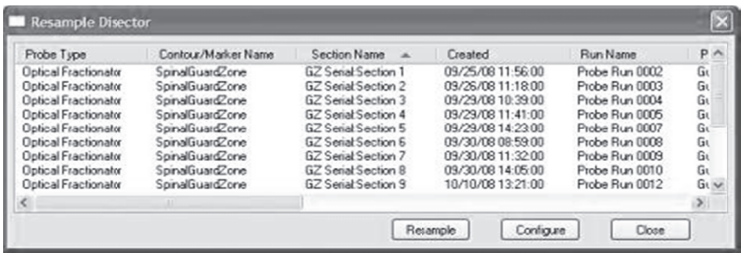
The Resample Disector probe is designed to identify the effect of tissue perturbations in cell counts in the Z dimension. It can help you to identify the appropriate guard zones and disector height.

Prerequisites

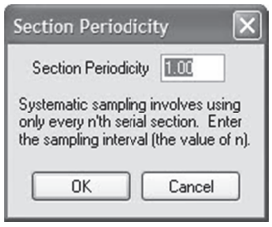
- You will need at least one complete probe run or have a probe run in progress.
- You must have Microsoft Excel 2003 or later installed to use this command.

To perform a Resample Disector probe run

1. Click **Probes>Resample Disector**. Stereo Investigator displays the **Resample Disector** dialog box.



2. Select the probe runs you wish to resample. Press SHIFT to select a series of contiguous probe runs. To select non-contiguous probe runs , press and hold down CTRL and then select the probe runs you want. Click **Resample**. Stereo Investigator displays the **Section Periodicity** dialog box.



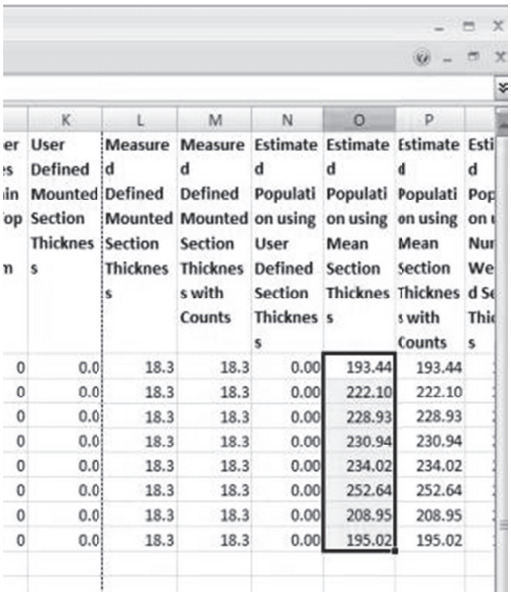
3. If you wish, change the periodicity and click **OK,** or just click **OK**. Stereo Investigator begins the resample oversample process. When complete, Stereo Investigator opens a Microsoft Excel workbook with the results.

Results

Stereo Investigator opens a Microsoft Excel workbook with your results in the following tabs:

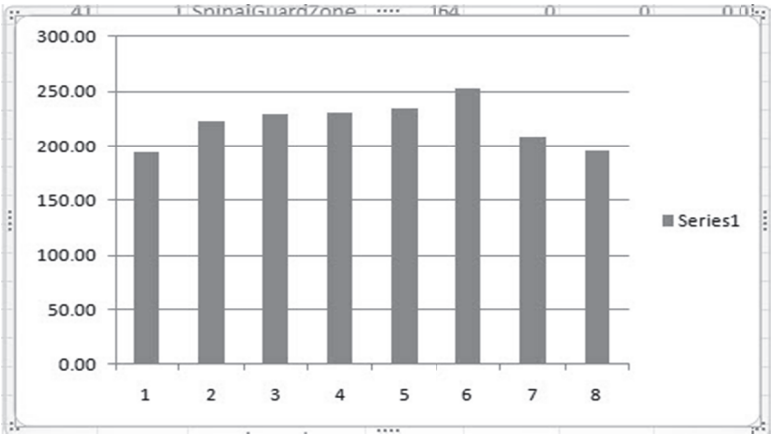
- Minimum Measured Disector
- Mean Measured Disector

1. Select the **Minimum Measured Disector** tab.
2. Select one of the columns labeled Estimated Population...that involved Section Thickness, and select the values, as shown below:



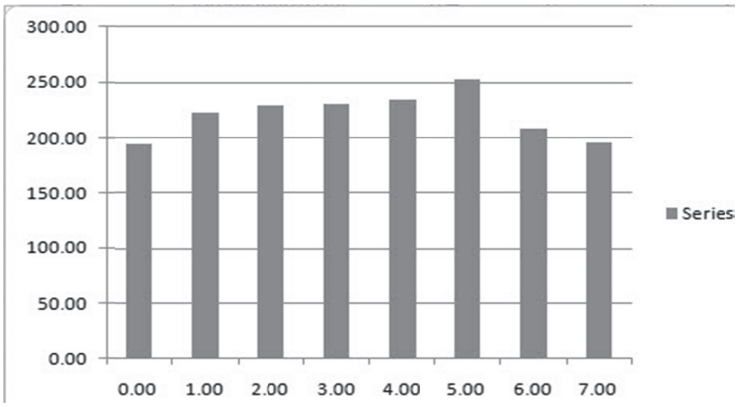
K	L	M	N	O	P
User Defined Mounted Section Thicknes s	Measure d Defined Mounted Section Thicknes s	Measure d Defined Mounted Section Thicknes s with Counts	Estimate d Populati on using User Defined Section Thicknes s	Estimate d Populati on using Mean Section Thicknes s	Estimate d Populati on using Mean Section Thicknes s with Counts
0.0	18.3	18.3	0.00	193.44	193.44
0.0	18.3	18.3	0.00	222.10	222.10
0.0	18.3	18.3	0.00	228.93	228.93
0.0	18.3	18.3	0.00	230.94	230.94
0.0	18.3	18.3	0.00	234.02	234.02
0.0	18.3	18.3	0.00	252.64	252.64
0.0	18.3	18.3	0.00	208.95	208.95
0.0	18.3	18.3	0.00	195.02	195.02

3. In Microsoft Excel, click **Insert>Column** chart, and select the first chart-type shown. Microsoft Excel inserts a column chart of your data.



The Y axis is your data. To include an X axis of the top guard zone values, right-click the numbers in the chart's X axis, and choose **Select Data**.

4. In the **Select Data Source** dialog box, click **Edit** under **Horizontal**.
5. Click-drag to select the values in the column you want to graph against. Microsoft Excel fills these values into the Axis Labels dialog box.
6. Click **OK**. Click **OK** again in the **Select Data Source** dialog box. Microsoft Excel changes the chart.



References

- L. Slomianka and M. J. West(2005) Estimators of the Precision of Stereological Estimates: An Example Based on the CA1 Pyramidal Cell Layer of Rats. Neuroscience 136(3), 757–767

Restart Z Scan

This feature is useful while you are using the Optical Fractionator in Automatic focus mode. It allows you to re-scan the 3D counting frame at the current sampling location. Stereo Investigator moves the focal depth to the beginning of the 3D counting frame, and the scan restarts. You can use it to check your work.

You can turn off display of the marker you are using to mark cells, select a different marker and then use this feature to remark, or have a co-worker mark, each cell as it comes into focus to double check your results.

Show All Probes

Displays all the probes in the Probes menu, overriding the settings of the Probes tab in Stereology Preferences.

Chapter 19

Menu Commands

File Menu

New Data File

This command starts a new tracing. Use this operation if you have already traced data on the screen and want to remove that tracing from the screen to begin a new tracing.

Open Data File

Opens an existing data file. Data can be in the MBF Bioscience ASCII file format (.asc), or the Neurolucida Explorer .nrx format.

Options

When opening a data file, you can choose from these options:

- **Merge**—Merges the data file currently open with the file you are opening. If you choose this option, **Close Currently Open Images** and **New Reference Point** are unavailable
- **Close Currently Open Images**—Closes any images open.
- **New Reference Point**—You must place a new reference point before Stereo Investigator opens the data file.
- **Load Images with Data File**—Loads images associated with the data file.

Open Files into Serial Sections

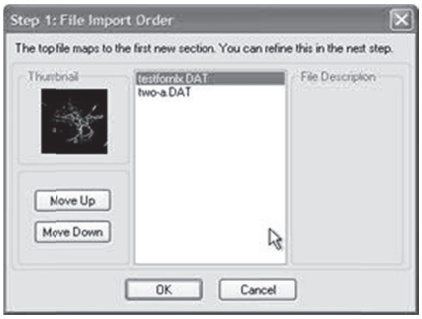
Use to move to move data not currently associated with a section into a new section. You can select of multiple files. For each file selected, Stereo Investigator creates a new section and all data from the file (that is not currently associated with a section) is put into the new section. For example, if you select 10 files Stereo Investigator creates 10 sections.

If Your Files Have No Sections Defined

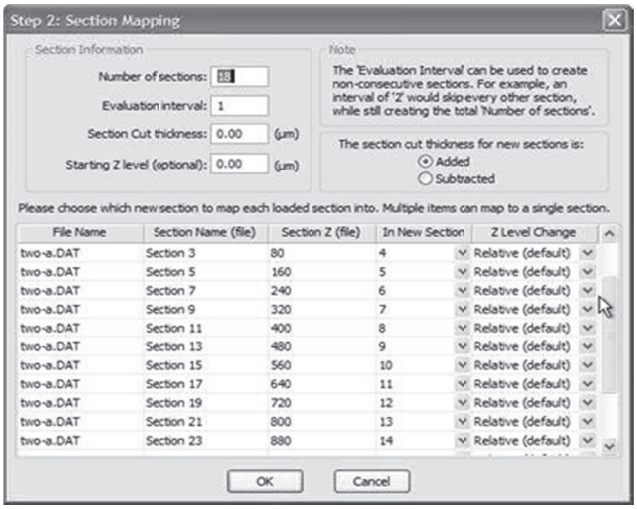
The instructions here are for the most simple case, when each section is saved to a separate file, with no sections defined, and you want to place all data from each file into a single section.

To Open a File with No Serial Sections Defined

1. Click **File>Open Files into Serial Sections**. Stereo Investigator displays the **Open File** dialog box.
2. Use the **SHIFT** and **CTRL** keys to select all files to be opened into sections, and then click **OK.** Do not check the **Merge** check box. Stereo Investigator displays the **File Import Order** dialog box.



3. To arrange the files in the desired order, select a file in the list and click **Move Up** or **Move Down.** When complete, click **OK.** Stereo Investigator displays the **Section Mapping** dialog box.



4. Enter the requested information. When complete, Click **OK.** Stereo Investigator loads the sections and displays the first section on screen.

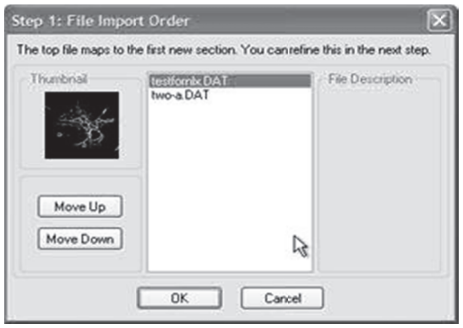
If Your Files Have Defined Sections

If there is data that is not associated with a section, use **File>Open Files into Serial Sections** to generate a new section where the unassigned data is placed. If you do not want to generate a new section for unassigned data, use **File>Open Data File** to open the first file, as described here.

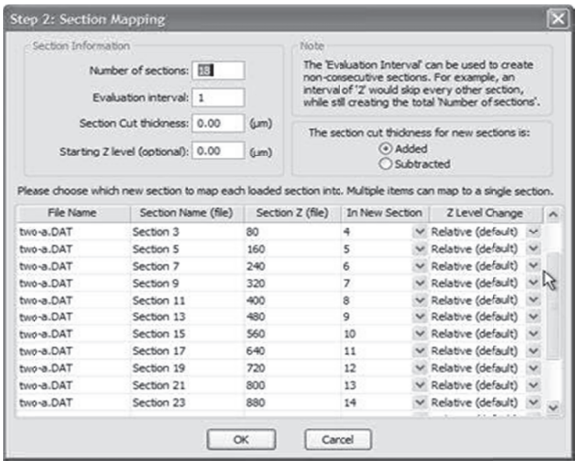
To Open the First File Without Generating a New Section

1. Click **File>Open Data File** to open the file with defined sections first.

2. Click **File>Open Files into Serial Sections** and select the other files open into the first file. Stereo Investigator displays the **New Section Order** dialog box. **Be sure to click Merge, otherwise the first file is automatically closed!** Stereo Investigator displays the **File Import Order** dialog box.



3. To arrange the files in the desired order, select a file in the list and click **Move Up** or **Move Down**. When complete, click **OK.** Stereo Investigator displays the **Section Mapping** dialog box.



4. Use the **Section Mapping** dialog box to set the Z value of the new sections. This determines where they are placed in relation to the existing sections. If they are in the middle of the existing stack of sections, they are inserted at the appropriate Z value. This may interrupt the order of existing sections if new sections are inserted in the middle of a stack, but all Z values are preserved.

5. When complete, Click **OK.** Stereo Investigator loads the sections and displays the first section on screen.

If data was not associated with a section in the original file, Stereo Investigator places it into a section with the same name as the original file name. Stereo Investigator merges previously existing sections with the same Z value into a single section. However, newly generated sections are not merged with other sections, even if they have the same Z value.

If files with all tracings already in existing sections are opened using **File>Open Files into Serial Sections**, an empty section with same name as the file is defined. For example, if a file called "File A" has 10 sections called sections 1-10, and it is opened with Open Files into Serial Sections along with a file called "Section 11", the new file will have 12 sections; Sections 1-11, and a section called "File A" that has nothing in it, since all the data in File A was already in a section.

Save Data File/ Save Data File As

Save Data File saves the file without asking for a filename, if the file already has a name.

Use **Save Data File As** to save a file with a new name, the same name but with different options, or with a different file type.

Other Save Options

To enable **AutoSave**, choose **Enable Auto Save** from **Options>General Preferences>AutoSave** tab. To save an image path along with the data file, check the option **Load Images with Data File** in the **Options>General Preferences>Imaging** tab.

Export Tracing

Exports your tracing for use in other programs. The file format determines the portion of the file that Stereo Investigator exports.

Export Formats

The following graphics formats export only the portion of the tracing shown in a raster or bitmapped format:

- .bmp (Bitmap file format)
- .eps (Encapsulated Postscript format)
- .jpg (JPEG file format)
- .pcx (ZSoft format files)
- png (PNG files)
- .tga (TARGA file format)
- .tif (Tagged Image File Format files)

If you want to capture the entire tracing in one of these formats, you can use the **Where Is** function to show the entire tracing in the current screen, then use **File>Export Tracing** to export this image.

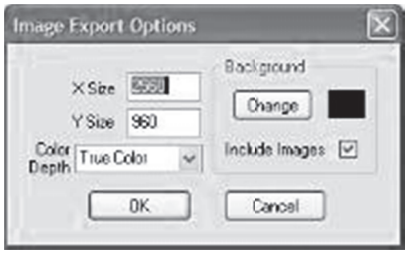
To export the entire tracing, select one of these formats:

- .wmf (Windows Metafile Format)
- .emf (Enhanced Windows Metafile Format)
- .dxf AutoCAD)

The resolution of the image captured in these file formats is determined by the resolution of the current screen image. Therefore, if you are viewing the tracing with a high magnification lens selected, or if you have zoomed in on a portion of your tracing, the entire tracing is exported at high resolution. This means that it also appears larger in the destination program, but can be rescaled while preserving detail. However, if you export while viewing the tracing at a low magnification lens or zoomed out, detail is lost, and the image appears smaller in the destination program.

To export a tracing

1. Click **File>Export Tracing**. Stereo Investigator displays the **Export Tracing** dialog box.
2. Type a filename and select a type, and then click **Export**.
   Depending on the type you selected, Stereo Investigator displays a dialog box.
   - For .bmp, .eps, .jpg, .pcx, .png, .tga, or .tif files, Stereo Investigator displays the **Image Export Options** dialog box.



   Make any modifications to the options. You can change the X and Y size of the export, Color Depth, and Background Color. If you want to export displayed images with the tracing, check **Draw Images**.

   - For .wmf or .emf files Stereo Investigator displays the **Export DPI** dialog box.

   - Make any modifications to the options. You can change the file's resolution. If you want to export displayed images with the tracing, check **Draw Images**.
3. Click **OK**. Stereo Investigator exports the tracing.

Image Open

Use to open bitmapped image files to view and trace. Images open into the upper left corner of the current tracing window

To open an image

1. Click **File>Image Open**. Stereo Investigator displays the **Image Open** dialog box.
2. Select a file from the list. You can also click the File name drop-down arrow and select from a list of recently opened files; you can also select the types of files listed with the Files of type list. Click **OK**.
   -or-
   Type or select an MBF Bioscience Image server and click **Go.** Stereo Investigator displays the **Image Scaling** dialog box.

3. Click **OK** to load the file with the default scaling.

To load an image file and change scaling

1. Load the image file.
2. When Stereo Investigator displays the **Image Scaling** dialog box, click **Change X and Y scaling**.
3. Change the **Source of Scaling**.
4. Change the **X** and **Y** values.

Scaling: Bitmap image files are not scaled according to the current lens. Image size and scaling is determined by the scaling in effect at the time the image was created. Therefore, before you trace data from acquired images that were previously acquired using Stereo Investigator, select the lens in use when the image was acquired. If the image was not acquired using Stereo Investigator or with your lenses, you should have a calibration image acquired under the same conditions so that you can calibrate a lens specifically for the magnification of the image file. See the Calibration topic for more details.

1. If you import an image with scaling that does not match an existing lens, Stereo Investigator alerts you to this, and asks if you want to define a new lens.
2. Click **No** to load the file, which is rescaled according to the current lens settings. Click **Yes** to define a new lens. For information on how to define a new lens, see Define a Lens.

The image always loads with the current lens active. If you have defined a lens for this image, you should change to that lens once Stereo Investigator loads the image.

Image Save / Image Save As

If you modified an image associated with a bitmapped file, use this command to save the image back to the original file. To save it to a new file, use **File>Image Save As**.

If you acquired a video image with Stereo Investigator or read in an image from another program, **File>Image Save As** lets you to save this image to a new file.

File Formats available

- Bit Map files (*.bmp)
- JPEG files (*.jpg, *.jpeg)
- PNG files (*.png)
- TARGA files (*.tga)
- Tagged Image File Format (*.tif, *.tiff)
- Portable Image Files (*.pgm, *.pbm, *.ppm).

Image Save As does not support black and white images; if you have a black and white image that you want to save, convert it to grayscale using Image Effects.

Saving images with Save and Save As

To save an image file

- Click **File>Image Save**. Stereo Investigator saves the file.

To save an image file with a different name or format

1. Click **File>Image Save As**.
2. In the **Save Image As** dialog box, you can type a new name, choose a different file type, and depending on the file type change compression.
3. Click **OK**. Stereo Investigator saves the file with your new options.

Compression

Image Save As may be used to apply image compression before saving an image. Click the checkbox or use the slider to set image compression.

If you are working with image stacks, only the currently visible image can be saved with this command. Use File>Image Stack Save As to save an entire stack with a new name.

Image Save /Image Save As in Virtual Image Mode

When saving an image in Virtual Image mode, you only save the part of the image that was retrieved for display on screen. Most image files supported by Virtual Image mode have each image saved using multiple resolutions. The part of the image saved, and the resolution of the image saved are dependent on what is being viewed and what zoom level is being used to display it. Think of any image you save as a snapshot of the current view of the Virtual Image. This partial image can be saved separately and used in documents, but after saving, it has no connection to the Virtual Image file or data file.

Choosing Resolution for Images Saved from Virtual Images

When saving, you need to choose the resolution for the saved image, using the **Part of Image** dialog box.

Stereo Investigator highlights the resolution closest to the displayed image. Accept this choice or choose another resolution, and then click **OK**. Stereo Investigator saves the image in the selected resolution.

Image Stack Open

This menu item is only available if the Image Stack module of Stereo Investigator has been purchased.

Lets you open an image stack or a stack of confocal images If you are opening a confocal image stack, you can simply select the file after choosing the appropriate file extension in the Files of Type field, and the entire stack is loaded into the Stereo Investigator image memory.

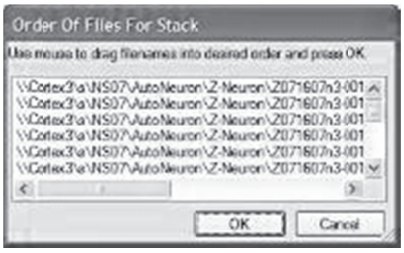
Tell me about image stacks

An image stack combines multiple images into a single image file or collection. Image stacks are stored in two distinct ways. The images that make up a single stack can be stored in a single multi-image file using two different file formats. . The PIC format is a BioRad proprietary format. The TIF format is a non-proprietary format that also supports multiple images in a single file. Fluoview files are a type of TIF file.

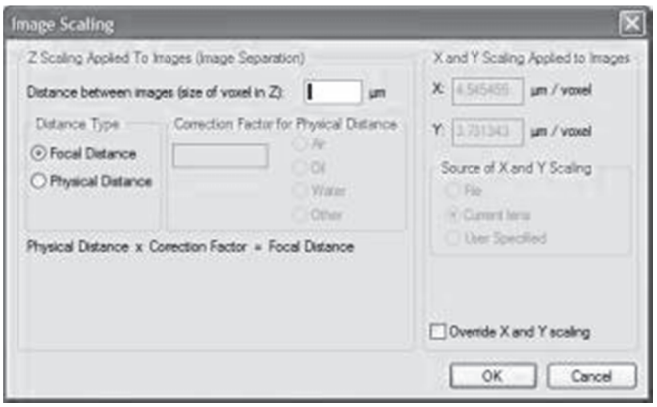
An alternative method stores the images in individual files, and load them in order using the Image Stack Open command. Some image stacks contain scaling information that Stereo Investigator uses to determine the distances between images. If your stacks don't have this information, you can tell Stereo Investigator the distances to use.

To load an Image Stack that consists of multiple separate image files

1. Click **File>Image Stack Open**. Stereo Investigator displays the **Image Stack Open** dialog box.
2. Select the images and click **Open**. Stereo Investigator displays the **Order of Files for Stack** dialog box.



3. If the files are not in the proper order, you can drag them in the list until the order is correct.
4. Click **OK**. Stereo Investigator displays the **Image Scaling** dialog box.



Since single image files don't contain Z spacing information, you need to manually enter this information. The program prompts you to enter the image separation while loading the stack. This is the distance between images. You can use the focal distance or the physical distance.

- **Focal Distance**—Image stacks collected with Stereo Investigator are collected using spacings that describe the focal plane separation.
- **Physical Distance**—describes the physical movement of the microscope stage as images are collected. If you select this option, correction factors must be applied to convert the microscope movement into the movement of the focal plane. The X and Y dimensions of the imported image default to the micron/pixel ration for the current lens. Select the lens that was used to capture the images before loading the image stack. If the image stack was collected on a different microscope it is important to calibrate a lens for that system. Select that lens before loading the image stack. For more information, please refer to the section Calibration for Imported Images.
  If you select this option, you need to select the correction factor for the physical distance between the lens and the image. Stereo Investigator automatically enter this value for **Air, Oil,** and **Water**. If you select **Other,** you must manual enter the factor.

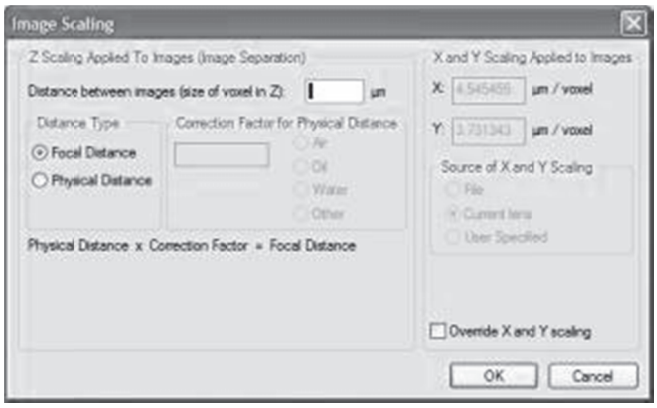
5. You can use the X and Y scaling used when the image was acquired, or override it. Click Override Z and Y scaling, choose the source, and then enter the values.
6. Click **OK**. Stereo Investigator loads the image stack.

If you load an image for which there is no matching lens, Stereo Investigator prompts you to define a new lens to match the image scaling. For information on defining a lens, see Defining lenses on page 18.

See the section Serial Sections and Imported Image Files on page 77 for more information on navigating and tracing using confocal image stacks.

Load an image stack that contains all the images

1. Click **File>Image Stack Open**. Stereo Investigator displays the **Image Stack Open** dialog box.
2. Select an image file, and click **Open**.
3. Click **OK**. Stereo Investigator displays the **Image Scaling** dialog box. Stereo Investigator needs this information if it isn't contained in the image file.



The program prompts you to enter the image separation while loading the stack. This is the distance between images. You can use the focal distance or the physical distance.

- **Focal Distance**—Image stacks collected with Stereo Investigator are collected using spacings that describe the focal plane separation.
- **Physical Distance**—describes the physical movement of the microscope stage as images are collected. If you select this option, correction factors must be applied to convert the microscope movement into the movement of the focal plane. The X and Y dimensions of the imported image default to the micron/pixel ration for the current lens. Select the lens that was used to capture the images before loading the image stack. If the image stack was collected on a different microscope it is important to calibrate a lens for that system. Select that lens before loading the image stack. For more information, please refer to the section Calibration for Imported Images.
  If you select this option, you need to select the correction factor for the physical distance between the lens and the image. Stereo Investigator automatically enter this value for **Air, Oil,** and **Water**. If you select **Other,** you must manual enter the factor.

4. You can use the X and Y scaling used when the image was acquired, or override it. Click Override Z and Y scaling, choose the source, and then enter the values.
5. Click **OK**. Stereo Investigator loads the image stack.

If you load an image for which there is no matching lens, Stereo Investigator prompts you to define a new lens to match the image scaling. For information on defining a lens, see Defining lenses.

See the section Serial Sections from Imported Image Files for more information on navigating and tracing using confocal image stacks.

Image Stack Merge and Open

This menu item is only available if the Image Stack module has been purchased.

Image Stack Merge and Open loads multi-channel confocal image stacks. These are image stacks that represent the same image captured with different filters or wavelengths.

Confocal image stacks that were saved as multi-channel image stacks must be opened using **File>Image Stack Merge and Open** to load the images properly. Loading a multi-channel image stack using **File>Image Stack Open** loads the images without merging.

Open Multi-Channel Image Stacks

To open a single, multi-channel image stack with each channel displayed in a different color, select **File>Image Stack Merge and Open**, and select the multi-channel file from the **Open Image Stack** dialog box. Stereo Investigator displays the **Select Desired Color Channels** dialog box.

If a multi-channel image stack is selected, the same file name appears in each of the Confocal Stack fields. Use the **Image Channel** fields to specify which channel appears in Red, Green, or Blue (as indicated by the color name at the left of the dialog box). Any color channel can be left blank by selecting none from the **Confocal Stack** field.

Merge Multiple Single Channel Image Stacks:

To open and merge multiple, single channel image stacks, use the SHIFT key and the left mouse button to select all desired image files from the **Open Image Stack** dialog box. The **Select Desired Color Channels** dialog box appears.

In this case, designate the color for each separate file by selecting the different image file names from the **Confocal Stack** fields. The Image Channel fields should remain blank, as each file contains only one channel. Any color channel can be left blank by selecting none from the Confocal Stack field.

View Multi-channel Image Stacks

the image files have been opened and merged, use the function keys to control which channel you are seeing. The **F9** key shows all channels, **F10** shows Red only, **F11** shows Green only, and **F12** shows Blue only. Use **PageUp** and **PageDown** to navigate up and down through the stack.

Image Stack Save / Image Stack Save As

Image Stack Save saves the image stack with its original name and location, overwriting the original image stack.

Use **File>Image Stack Save As** to save the modified stack without altering the original file.

Options

You can choose the following options:

File options

- **Save as type /Export as type:** Choose the file type from the drop-down lost.
- If you want to save just the image without the marker or tracing data, click **Export the image file.**

Stack Save Options

- **Save a stack as series of single image files:** If you want to save each image as a separate file, click **Save as a series of single image files**. When you save the file, Stereo Investigator adds the number 001, 002, 003, and so on to the file name. For example, "neuron_ly_40x.tif" is saved as *neuron_ly_40x_001, neuron_ly_40x_002, neuron_ly_40x_003,* and so on.
- **Save a stack as red, green, and blue image stacks:** You can save the files as red, green, and blue image stacks. Click **Save as a series of single image files**. When you save the file, adds the R, G, and B to the file name, indicating the channel. For example, "neuron_ly_40x.tif" is saved as *neuron_ly_40x_R, neuron_ly_40x_G, neuron_ly_40x_B*.
  If you choose **Export the image file** option, this option changes to **Save as individual channels.** The Save image color option becomes available when you choose this option. This saves the color information with each separate channel or stack.

Images for Montage Open

You can load images with the **File>Images for Montage Open** command or with drag and drop.

To open a set of images

1. Click **File>Images for Montage Open**. Stereo Investigator displays the **Image Open** dialog box.
2. Click a file name. Hold down CTRL to select non-contiguous files. Hold down the SHIFT key to select contiguous files.
3. Click **Open**. Stereo Investigator loads the files into your workspace.

Montage Save As

You can save image montages as images or as DAT files. When you save a montage as an image, you "flatten" all the images into one image—you can't manually move or realign the individual images after saving as a new image. When you save as a DAT file, you save the files and layout structure so that you can later open the DAT file and work with the image montage and its tiles.

To save image montages

- Click **File>Save Data File** or **File>Save Data File As** to save as a data file.
  or
  Click **File>Montage Save As** to save the new montaged image.

Close All Images

Closes all open image files. If you have made any unsaved adjustments or changes to an image file, Stereo Investigator prompts you to save the file or discard the changes.

File Description

Use **File Description** to add notes about the file.

Print

Prints a hard copy version of the tracing on any of the printer devices installed under Windows. You can print:

- **Tracing on Screen**—Prints only the current contents of the tracing window
- **Tracing and Images on Screen**—Prints the tracing with displayed images.
- **All Tracing**—Scales the entire file to fit the paper size
- **All Tracing with Images**—Scales the entire file including images to fit the paper size.

Most printers have a higher resolution than a computer monitor. If you display markers sized in pixels, they may not print at the same relative size as you see on the monitor. Click **Options>Display Preferences>Markers Tab** and set **Marker Sizing** to *In Microns*, so that the relative size of the markers on the screen and on the printed page is the same.

Print Preview

Previews the graphics that are used to produce a hard copy of your tracing on any of the printer devices installed under Windows. You can print:

- **Tracing on Screen**—Prints only the current contents of the tracing window
- **Tracing and Images on Screen**—Prints the tracing with displayed images.
- **All Tracing**—Scales the entire file to fit the paper size
- **All Tracing with Images**—Scales the entire file including images to fit the paper size.

Recent Data Files/Recent Image Files

Displays a list of the four most recently opened data file or image files. Click on a file name to open the file.

Exit

Ends the current session. If you have any unsaved work, Stereo Investigator prompts you to save it.

Edit Menu

Undo

Undoes many tracing and editing operations. This command is not available if all contours are finished.

Select Objects

Starts the Editing Mode. Once in the Editing Mode, active tracing is disabled, and objects can be selected and altered using the features of the Editing Mode. For more information, please see Selecting objects.

Select All Objects

Starts the Editing mode and selects all objects on screen for editing.

Reveal Hidden Objects

Reveals any objects you have hidden.

Reveal Hidden Objects does not restore the hidden objects, but displays them in a khaki color to let you see their location. To restore hidden objects, you need to enter the Editing Mode via **Edit>Select All Objects**, or select all hidden objects, right click, and choose **Restore Hidden Objects**.

Paste Objects

Pastes objects into the same location from which they were copied, so they will overlay the original objects if all sections are visible.

Objects cannot be pasted into the same section from which they were copied.

3D Reconstruction

When this tool is used, all of the section tracings are moved to the Z depth of the section in which they are contained. Any tracings not contained in a section are left at their original Z depth value. This tool is used to convert a Stereo Investigator file into a format that can be used to generate a 3D reconstruction in Neurolucida Explorer. In a typical Stereo Investigator file, the tracing data for each section is started at a Z depth of zero, regardless of the position of the section. That is, the section Z information is used to calculate volumes and estimate final values, but is not incorporated into the display of the section tracings. With the 3D reconstruction tool, the Z values are adjusted to match the designated Z depth for each section.

Copy to Clipboard (BMP)

Copies the tracing to the Windows Clipboard.

If Stereo Investigator is displaying an acquired image, it is sent to the Clipboard along with the tracing. This way, images of the specimen with the tracing overlaid can be acquired for printing or further processing.

To copy the tracing without the acquired image, turn off **Imaging>Display Acquired Image**, or click **Imaging>Live Image**.

Copy to Clipboard (Metafile)

Copies the tracing to the Windows Clipboard.

After clicking **Copy to Clipboard (Metafile)**, Stereo Investigator displays the **Export DPI** dialog box. Set the DPI of the copied tracings. Higher DPI settings capture more detail. To capture images in the tracing window, select **Draw Image** in the **Export DPI** dialog box.

The Metafile format maintains contours, markers and text as separate vector objects that can be selected and altered in other graphics programs. For best results when pasting to a graphics program, set the DPI of the current file in that program to match the DPI selected in the **Export DPI** dialog box.

Paste from Clipboard

Pastes data from the Windows Clipboard into the tracing window.

Add Text

Use **Add Text** to place a text label at any position on the tracing. Text labels are useful for helping explain your tracings and data. Stereo Investigator make it easy to add, move, and modify text labels.

You can add text anywhere on the tracing.

To add text

1. Click **Edit>Add Text.**
2. Click a point in the window to place the text. Stereo Investigator displays the Add Text dialog box.

3. Type the text. The text is centered as a single line. You can also click **Keyboard** and use the mouse to type characters. This is useful when using a Lucivid with Stereo Investigator.
4. Click OK. Stereo Investigator displays the text.

5. Click Set Font modify the text font, size, and color.

Edit the text

If you make a mistake or if you want to change the text, you can edit it.

To edit text

1. Click on the text to select it, and then right-click.
2. Choose **Change text.**
3. In the **Modify Text** dialog box, type the new text or make corrections, and then **click OK.**

Change text characteristics

You can change the font, color, it's Z Position, and apply shrinkage to the text.

To change your text characteristics

1. Click on the text to select it, and then right-click.
2. Choose one of the following commands and follow the on-screen instructions.
    - Change Text to color—Pick a new color from the **Color** dialog box.
    - Change Font—Pick a new font from the **Font** dialog box.
    - Modify Z Position—Set or Shift the Z position for the text.
    - Apply Shrinkage—Displays the **Shrinkage Correction** dialog box, which you use to set the X, Y, and Z positions of the text.

Manipulate text

In addition to changing the text characteristics, you can manipulate the text for other uses or effects. You can move it, rotate it, hide it, delete it, copy it, and place it into a set of tracing objects

To manipulate text

1. Click on the text to select it, and then right-click.
2. Choose one of the following commands.
    - Rotate text—Rotates text, with the center of the line as the axis. This does not rotate the text 360 degrees around the center, rather, it uses the center of the text as the point on which the entire line of text revolves. The text is always horizontally aligned.
    - Flip text—Displays the Flip Selected dialog box. You can choose Horizontal (X) or Vertical (Y). You can also use the reference point as the origin of flipping point
    - Delete text—Deletes the text.
    - Hide text—Changes the text into a hidden object. Use **Edit>Reveal Hidden Objects** to view the text.

- Copy text—Copies the text. Right-click and choose Paste objects to drag the copied text on screen and place it.
- Place text into set—Pleases the text into an existing object set or into a new set.

Undo your changes

There are two ways to undo an action or changes you've made.

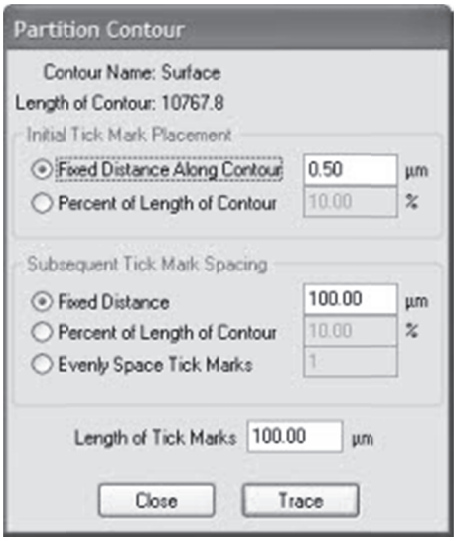
- Right-click and choose Undo to reverse the last action.
- Click **Edit>Undo** to undo the last action. Click again to undo the next, and so on.

Partition Contour

This command places evenly spaced tick marks on open contours to partition them into segments.

To Use Partition Contour:

1. Click **Edit>Partition Contour**. The tracing window displays an overview of your work so that you can see all of your tracings. Stereo Investigator adds a white box to the ends of all open contours (for selection), and displays the Partition Contour dialog box.



2. Select a contour.
3. Specify the initial mark placement, spacing, and length of the marks.
4. Click on an open contour's ending box.
5. Click **Trace**.

You can't undo a Partition Contour, except by removing each of the tick marks manually in the Editing Mode.

Mark Contour Centers

Places a marker at the center of each contour.

Probes Menu

For information on using the probes, please see Stereological Probes: Foundations

Move Menu

Center Last Point

Centers the last point drawn in the tracing window.

Move To

Use to move your tracing and slide specimen/image a short distance within the tracing window. This operation allows for registered movement of both the tracing and the specimen to a new location.

To move to

1. Click **Move>Move To**.
2. Click a point on the overlay.
3. Click a point where the image and tracing should be moved to (appear). You may need to refocus.

This option is useful if you want to move your specimen and tracing only a short distance. For larger movements, use **Go To** or the Field commands (from the **Move** menu or using **the Field Movement** arrow keys on the **Movement** toolbar).

To Reference Point

Centers the reference point in the tracing window.

To Counting Frame

Centers the counting frame over the tracing window.

Where Is

Use **Move>Where Is** to find the current field-of-view relative to the entire tracing. In **Where Is** mode, live video is temporarily turned off and the tracing window zooms out so that all parts of the tracing are visible. All contours and neuronal processes are rescaled, although the size at which markers display may not change. Imported or acquired images (bitmaps) are still visible. The **AutoMove** box disappears. Live video and the **AutoMove** box are restored when you exit **Where Is**.

In **Where Is** view, a dashed rectangle shows the current field of view, allowing you to see where the current location is with respect to the entire tracing. If the current field-of-view is very small, the box around it blinks to make it easier to locate.

Zoom in to magnify any portion of the tracing that is displayed in the **Where Is** view by holding down the CTRL key while dragging down and to the right with the left mouse key depressed. The cursor

changes to a magnifying glass. Zoom in as many times as you want. To zoom out, right click and select **Zoom Out**.

To return to the normally scaled view, left click anywhere in the trace window, click the **Where Is** button on the **Main** toolbar, or deselect **Where Is** from the **Tools** menu, or press the ESC key. Stereo Investigator returns to whatever task was active before the **Where Is** mode was activated.

Go To

Changes the tracing window to a large **Macro View** window, showing an overview of the entire tracing. Click any point to return to the tracing window with that point centered. The stage automatically moves to that point to realign the tracing and specimen. Use the ESC key to cancel a **Go To** operation.

If you want to see a part of the tracing at higher power before choosing a location to center, hold down the CTRL key while dragging a box around the area to be magnified. Click on a point in the magnified image to center that point of the tracing and specimen and return you to the magnification of the current lens.

Joy Track

Use **Joy Track** to move the stage with the joystick, and have Stereo Investigator track the movements. Remember that **Joy Track** keeps track of movements in X, Y and Z. To focus without changing the Z position of the tracing, remember to first activate **Joy Free**.

Use the right-click menu to switch to between Joy Free and Joy Track.

Move Images and Tracing

Use **Move Image** and **Move Images and Tracing** to move acquired images.

- To move the image with the tracing, select **Move Images and Tracing**.
- To move the image while leaving the tracing stationary, choose **Move Image**.

If the image does not move, open the Image Organizer to be sure that the image is checked. Only checked images are moved. If multiple images are checked, they are moved together.

Fast Focus

Use the **Fast Focus Adjustments** dialog box

to move the stage up and down. Click an arrow or arrow set next to the desired distance to move the stage.

Warning: Remember that focusing in the negative direction, or down through the tissue, moves the stage closer to the objective lens; be sure that you have adequate clearance before attempting large movements in this direction or you can break the slide!

If you have a focus motor installed, focusing is usually done by **Fast Focus** or a knob on the joystick. Do not use the fine focus knob on the microscope if you have a focus motor, as this can strip the gears of the focus motor. You can use the coarse focus on the microscope, but keep in mind that these movements in the Z-axis are not recorded by Stereo Investigator unless you also have a focus position encoder. If you are doing a 3D reconstruction or a multi-section neuron tracing, the Z-axis information is essential to the accuracy of your data. The general recommendation is: Unless you have an internal Z motor on your microscope do not use the focus knobs on the microscope at all beyond initial set-up of the slide on the microscope. If focusing with the joystick is too slow, check the specifications of your joystick; many have a switch that controls the speed of focus and have multiple settings. If you have an internal Z motor on your microscope, then the system is designed to be used with the microscope's fine focus knob, and the focus on the joystick is disabled.

Field (movement)

Use Field Movement to move your view up, down, left, or right. This command is equivalent to using the directional arrows on the Main toolbar of Stereo Investigator.

Set the amount of movement as a percentage of screen size with the **Options>General Preferences>Movement** tab.

Synchronize Stage and Images

When active, **Synchronize Stage and Images** moves the stage with the movements of an acquired image. For example, if you have acquired an image of a specimen, and are tracing or mapping from the image, **Synchronize Stage and Images** causes the stage to move each time you move the image. When you return to live video mode, the specimen and tracing are still aligned.

If not active, **Synchronize Stage and Images**, returns you to the last location of a live image when you switch back to live video mode.

This mode is very useful when working with tissues that are light sensitive, such as fluorescently stained tissues. It is possible to do the tracing from a series of acquired images while still using movement features such as the **AutoMove** window or **Meander Scan**, and maintaining the registration between the stage and the tracing.

Joy Free

Joy Free allows free movement with the joystick that is not tracked . Use **Joy Free** to move the stage to a new section on a slide, when placing a new slide, or when aligning new sections with previous tracings. Use **Joy Track** to move the stage with the joystick, track the movements, and realign the specimen and tracing.

Use the right-click menu to switch between modes.

Once you exit **Joy Free**, it is not possible to return to the previous alignment of overlay and specimen.

Joy Free or **Joy Track** disable all tracing and mapping functions. Exit the Joystick mode before returning to tracing.

Move Image

Use **Move Image** and **Move Images and Tracing** to move acquired images.

- To move the image with the tracing, select **Move Images and Tracing**.
- To move the image while leaving the tracing stationary, choose **Move Image**.

If the image does not move, open the Image Organizer to be sure that the image is checked. Only checked images are moved. If multiple images are checked, they are moved together.

Align Tracing

Align Tracing moves the tracing in reference to the specimen, letting you to align a new section with previous tracing.

Set Stage Z

Use to manually set the Z value for the stage.

To set the Z position

1. Click Move>Set Stage Z
2. In the Stage Z Position dialog box, enter a new value, and then click OK.

Meander Scan

Meander Scan is an automated scanning procedure that is used to ensure that all points within a closed contour are viewed by moving systematically through the contour. The directions that follow walk you through the steps for setting up and executing a **Meander Scan**.

To perform a meander scan

Use a low-powered lens to draw the contour. If your region of interest has contours within contours, **Meander Scan** treats the interior contours as an exclusion zone, and does not visit these. If you want to include these areas, select these interior contours, right-click and choose **Hide Selected Contours**.

1. Draw a closed contour around the region of interest.
2. Click **Options>General Preferences>Movement** tab. Set the Field Movement size to 75% of Screen Size, and click **OK**.
3. Click **Move>Meander Scan**. In the **Meander Scan** dialog box, click **Start Meander Scan**. If there is more than one contour, Stereo Investigator displays the **Macro View** window. Click inside the desired contour to scan.
4. Mark, trace, or map anything within the current field-of-view.

5. To move to the next scan site, click **Move>Meander Scan** and select **Next Scan Site**.
   -or-
   Right click in the tracing window and choose **Next Scan Site**.
   -or-
   Use the **Next Scan Site** button. If you think you missed something in a previous section, click **Previous Scan Site.**

Click **Move>AutoMove** if there are structures that extend beyond one field-of-view. If using **AutoMove**, we recommended that you return to the previous site before continuing mapping, just to be sure nothing was missed before **AutoMove** took you away from that scan site.

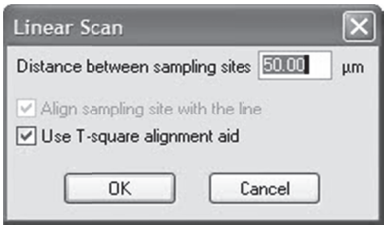
When you are viewing the last site, click **Move** to end **Meander Scan**.

Linear Scan

Linear Scan is very useful for working in cortical layers. This tool displays a series of counting frames arrayed at a set distance from one another along a straight line or linear feature. The straight line is set when the linear scan is started, so no previously traced contour is required.

To Use Linear Scan:

1. Click **Move>Linear Scan**. Stereo Investigator displays the **Linear Scan** dialog box.



2. Select the distance between each sampling site, whether to align the sampling site with the line, and whether to use the T-square alignment aid when defining the scan axis.
3. Click **OK**. Stereo Investigator displays an aerial view of the tracing.
4. Click on the beginning point for the scan.
5. Click on the ending point for the scan. **Stereo Investigator** moves you through sequential fields of view along the selected scan axis that are separated by the distance you selected.

To Previous Scan Site

Use this command with any of the scanning probes, such as the **Fractionator, Optical Fractionator, Linear Optical Disector,** etc.

This command allows you to return to and review the previous scan site. If a probe or scan is in progress, you may use this command to step backwards to each scan site that has been previously visited.

AutoMove

AutoMove is an automatic centering procedure for use with motorized stages. It acts in conjunction with a dashed rectangular boundary, known as the **AutoMove Area** to allow for the continuous tracing of

structures larger than a single field-of-view Click **Move>AutoMove** to activate it, or use the **AutoMove** button.

When active, **AutoMove** automatically centers the display when you click outside **AutoMove** window area. Both the tracing and the stage move in unison so that there is no loss in registration. Note that there may be a momentary delay as the stage moves to its new location. Continue tracing uninterrupted.

If the **AutoMove** area is defined "backwards", that is, by clicking the lower right corner first, then the upper left, each point that is drawn is centered immediately, whether it is inside or outside the **AutoMove** area.

AutoMove Settings

Set the **AutoMove Area** boundary to encompass the central two thirds of the screen. This reduces visual confusion when your stage executes a move to center a peripheral point.

To define the AutoMove Window

1. Click **Options>Define AutoMove Area**.
2. Click and drag from the upper-left to the lower right to define the **AutoMove** area. If you drag from lower-right to upper-left, each drawn point is immediately centered, whether inside or outside the **AutoMove** area.

Center Point

You can define the Center Point as either the center of the tracing window or the center of the AutoMove window. These are not necessarily the same, depending on where you places the **AutoMove** window. The center of the tracing window is the default. To center the point at the center of the **AutoMove** window, Click **Options>General Preferences>Movement** tab and check **Center Cursor in AutoMove Area**.

Define AutoMove Area

Use **Move>Define AutoMove Area** to define a rectangular on the screen. Messages on the Status bar describe the steps for this procedure. Position the cursor and click the left mouse button to mark the upper left-hand corner of the new **AutoMove Area** boundary. Move the cursor to the lower right corner and left click again. Press the SHIFT key and move the mouse (before selecting the lower-right corner) to change the position of the **AutoMove Area**. The SHIFT key allows the rubber box to move instead of changing the size of the box.

We recommend that you set the AutoMove Area boundary to encompass the central two thirds of the screen. That reduces visual confusion when the stage executes a move to center a peripheral point that is picked.

Use the ESC key or click **Move>Define AutoMove Area** to cancel the operation. The new **AutoMove Area** goes into effect as soon as the second corner is clicked.

Tools Menu

3D Visualization

The 3D Visualization command opens the 3D Visualization window where you can see your image and tracing in 3D. For more information, please see the 3D Visualization chapter starting on page 87.

Serial Section Manager

For information and instruction, see the Serial Section manager chapter starting on page 71.

Depth Filter

For information on the Depth Filter, please see the Orthogonal View on page 12.

Random Number Generator

Displays the **Random Number Generator** dialog box, which you can use to get a random number.

To Generate a Random Number

1. Click **Tools>Random Number Generator**. Stereo Investigator displays the **Random Number Generator** dialog box.
2. Enter the minimum and maximum values for the number. This must be a whole number; a decimal is not allowed.
3. Click **Apply**. Stereo Investigator generates and displays a random number.
4. Click **Close**.

Orientator

Displays the **Orientator** dialog box, which you use to generate random values for the Z axis in the XY Plane and in the place defined by the previous angle.

To use the Orientator

1. Click **Tools>Orientator**. Stereo Investigator displays the **Orientator** dialog box.
2. Click **Randomize**. Stereo Investigator generates random values you can copy to use.
3. Click **Close**.

Automatic Contouring

Starts the Automatic Contouring operation. Automatic Contouring lets you interactively trace live or acquired images, an often time-consuming and tedious task. Based on the parameters and options you set, Automatic Contouring will trace a contour and also move the stage to follow the image's contour. For more information and instructions, see Automatic Contouring on page 38.

Screen Snapshot

Takes a snapshot of everything displayed in the work area and adds the image to the Image Organizer. You can then save or discard the snapshot.

Quick Measure Functions

Use the Quick Measure tools to make instant measurements of objects in the tracing window. These tools can also be used to test lens calibrations and to do some fine tuning of the calibrations

Three different quick measurement options are available. You can use then any time during mapping or tracing. Each displays measurements directly. The measurements are saved and can be viewed, printed, or copied to the clipboard by selecting **Tools>Display Quick Measurements**.

Stereo Investigator doesn't save these measurements to the data file; they are only available during the current session.

Each of these measurement tools operates in a continuous mode, where you can take successive measurements until you right-click and choose **Stop Quick Measurements**. To take only one measurement at a time, deselect Continuous on the right click menu.

Quick measuring also stops if any other action is taken, i.e., **Align**, **Move To**, selecting a marker, etc.

The ESC key also stops any of the quick measurements.

Quick Measure Line

Quick Measure Line uses a rubber band line to measure the distance from the point first clicked to any other point. The length of the rubber band line is continuously updated in the status bar. It is possible to measure the distance across multiple fields of view by clicking on the arrow buttons to move the stage. It is not possible to move the stage using the **Move>GoTo or Joy Track** commands.

Pressing the SHIFT key allows the entire rubber band line to be shifted. If the CTRL key is pressed, the rubber band line movement is constrained to 22½° increments, which makes it easy to draw perfectly horizontal and vertical lines. Shifting a line makes it possible to place the starting point exactly.

To take multiple-length measures from the same point

The advantage of this technique is that all of the measurements are guaranteed to be taken from the same initial point.

1. Click **Tools>Quick Measure Line**.
2. Click on a point. The rubber line extends from the point to the cursor.
3. Position the cursor to take a length measurement.
4. Press **F8**. Stereo Investigator records a measurement, but the rubber line is still anchored at the initial point.
5. Move the cursor to another position.
6. Press **F8** again to take another measurement.

Quick Measure Circle

Click and drag the cursor to use **Quick Measure Circle**, which uses a rubber band circle centered on the point first clicked to measure the radius, diameter, and area of a circular region. The radius and area of the rubber band circle are continually updated in the status bar. When the next point is clicked with the left mouse button, displays the radius, diameter, circumference, and area of the circle.

Holding down the SHIFT key and moving the cursor moves the rubber band circle. The rubber band circle moves without changing size. This is a useful way to quickly compare circular sizes, for example, comparing cell sizes. The CTRL key has no effect on circle measurements.

Quick Measure Angle

Quick Measure Angle measures an angle defined by three points.

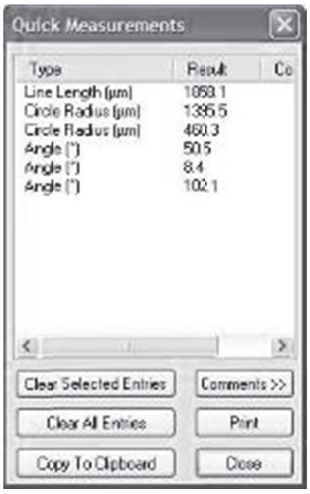
To use Quick Measure Angle

1. Click **Tools>Quick Measure Angle.**
2. Click along the first side of the angle.
3. Move the mouse to the vertex of the angle and click again. The angle formed by the rubber band lines is continuously updated in the message bar.
4. End by clicking on a point on the second side of the angle. A dialog box displays the angle in degrees and radians.

Pressing the SHIFT key allows the angle formed by the rubber band lines to be moved. If the CTRL key is pressed, the angle movement is constrained to 22½° increments.

Display Quick Measurements

Quick measurements for each session of Stereo Investigator are temporarily recorded for the duration of that session as well as being immediately displayed. To view the recorded quick measurements click **Tools>Display Quick Measurements.** The recorded quick measurements can be viewed, printed, or copied to the clipboard.



Display Quick Measurements displays measurements in the order in which they were made. The Type column lists Line Length, Angle, or Circle Radius. The Result column lists the most basic result gained

from the measurement without units. To see the more detailed results and units, double click an item in the Type column for the measurement of interest.

Clear Selected Entries

Clears all selected entries in the Type column. To select multiple entries, hold down the SHIFT key and click on contiguous entries you would like to select. For non-contiguous entries, or to deselect entries, hold down the CTRL key and click.

Clear All Entries

Erases the display, allowing new measurements to be gathered. All of the previous measurements are deleted.

Copy to Clipboard

Copies the current list of measurements to the Clipboard. Only the selected measurements are copied. If no measurements are selected, then all of the entries are copied to the clipboard.

Comments

Some measurements may be particularly interesting. It is possible to label these measurements with a comment. Click on the Comments button to expand the dialog box. Click one of the measurements by clicking in the Type column. Click in the entry field box to the right of the buttons and enter a comment. The comment is entered and attached to the measurement when you click in any other field of the measurement display table. Comments can be changed by reselecting the measurement, entering changes, and clicking on another field. Click on Collapse to reduce the size of the dialog box.

Print

This button prints out the current list of measurements. Only the selected quick measurements are printed. If no quick measurements are selected, then all of the entries are printed.

Close

This button closes the **Display Quick Measurements** dialog box.

Define New Lens

Stereo Investigator requires that one or more lenses are always defined. Lenses are defined in two steps. The first step is to describe the gross properties of a lens such as its name and type. The second step is the calibration step that fills in the details of the lens. Begin by entering the descriptive lens information for a given lens and click on OK. The calibration step follows immediately. The calibration squares of a graticule slide should be visible. Click on the upper left corner of a calibration square. Then click on the lower right corner of the same calibration square. A grid of dashed lines appears over the calibration squares. Stretch the grid until it matches the calibration squares.

The Define New Lens Dialog Box

Name

Enter a name for the lens. This is the name that appears in the **Lens Selection** list on the **Main toolbar**.

Each objective lens on the microscope has different calibration parameters according to whether it is used with a video camera or the Lucivid. It is helpful to choose a name that permits unambiguous

identification for each viewing mode. Remember that each resolution of the monitor also requires a different lens, so specify 'hi-res' or 'lo-res' (for example) if using the monitor at different resolutions.

Comments

You can add comments to help you identify the lens and its conditions of use. These comments are inserted into the lens calibration file, 'Stereo Investigator. len', and do not appear elsewhere. The comments are only displayed when editing a lens.

Units

The default units value is micrometers, but this can be changed to millimeters or nanometers (for example, for imported images or data tablet use).

Lens Type

Select one of the following:

- Optical: Optical lenses are used with the Lucivid. Select optical for lens that involve direct viewing through the oculars of the microscope
- Video: Video lenses are used with an analog or digital video camera.
- Tablet: The Tablet selection does not specify a true lens but indicates that a data tablet is used to acquire the data. The lens is calibrated by placing a scale bar on the tablet. This lens can also be used for all image stack, MRI, or other imported images.

Correction Factor

The correction factor is used to calculate the true position of the focal plane as the microscope is moved up and down. The focal plane moves a distance that may differ from the movement of the microscope. The difference is due to the refraction of light between the cover slip and the immersion medium of the lens. Under normal circumstances the predefined values for air, oil, and water immersion lenses can be used. There are circumstances in which the predefined settings should not be used. Contact MBF Bioscience for information about unusual combinations of lenses and mounting mediums.

Select one of the following:

- Air
- Oil
- Water
- Other

To enter a nonstandard correction factor use Other. The correction factor is usually the inverse of the index of refraction between the tissue and the lens immersion medium.

Calibration Box Setup

Box Size

Enter the dimension of one side of the square calibration box. This is the size of the squares on the calibration slide (25µm and 250µm on the MBF Bioscience calibration slide) or calibration bar that you are using.

Force Square

This feature was designed for use with acquired images where a calibration square is not available and calibration must be performed with a calibration line or bar. Do not use this option when calibrating with a 2-dimensional grid.

Grid Tune Current Lens

This option provides a very efficient method for lens calibration.

To fine tune calibration for a lens:

1. Place the MBF Bioscience graticule slide on the microscope and bring either the large or small graticule squares into focus.

   On most systems it is convenient to view the larger graticule squares when using objectives up to 10X and the smaller graticule squares with objectives more powerful than 10X.

2. Select **Tools>Grid Tune Current Lens**. The **Grid Tune Current Lens** dialog box is then presented.
3. The name of the currently selected lens is displayed in the Name field. Make sure this matches the objective lens in use on the microscope.
4. In the **Box Size** field, type in the size of a single graticule square visible on the screen or in the eyepieces. The MBF Bioscience graticule slide has squares that are 25µm and 250µm.
5. Click **OK**.
6. Dashed white lines which comprise the grid tuning pattern appear on the screen.
7. Place the cursor over the anchor symbol (found at the intersection of a horizontal and a vertical dashed line). The anchor disappears when the cursor is directly over it.
8. Hold down the left mouse button and drag the anchor's intersection to an intersection of two black lines on the graticule slide that is located near an edge of the screen. Position it carefully so that this vertex exactly matches this corresponding vertex of the graticule slide.

   Placing the first intersection near an edge of the screen maximizes the distance from the anchor along a given axis. A small error in one square is magnified over larger distances, so it is best to perform the calibration over as great a distance as possible. However, your image may show some optical aberrations near the edges, so be sure to only calibrate in the uniform region of the image.

9. Examine all the vertical dashed lines in relation to the vertical black lines of the graticule slide.
10. If some of the dashed lines do not lie exactly on the black lines, place the cursor on a vertical dashed line (using a dashed line farthest from the anchor works best); the cursor changes into a double arrow, pointing both left and right. This indicates the cursor is directly over the dashed line. Now hold down the left mouse button and drag this vertical dashed line to the left or right until it lies exactly on top of its corresponding black line on the graticule slide.
11. Make sure that all the dashed vertical lines lie directly on top of their corresponding black vertical graticule lines. If not, readjust them using this same technique.
12. Perform the same type of adjustment for the horizontal dashed lines relative to the black horizontal lines of the graticule slide.

13. When all the dashed lines (or at least those in the center third of the field-of-view) lie directly on top of their corresponding black lines on the graticule slide, the calibration settings for this lens have been properly fine-tuned.
14. With the cursor in the tracing window, click on the right mouse button to bring up the context sensitive right click menu. Move the cursor over the **Adjust Current Lens With Grid Tuning** entry and click on it with the left mouse button to complete the grid tuning procedure.

Parcentric/Parfocal Calibration

Parcentric/Parfocal Calibration compensate for the deviations from parfocality (focal plane) and parcentricity (collimation) that are normally encountered between different microscope objective lenses. For more information and procedures, see the Parcentric and Parfocal Calibration on page 25.

Edit Lens

Use this command to edit the properties of existing lenses already part of your Stereo Investigator system. For more information and procedures, see Working with Lenses starting on page 17.

Focus Step Size Calibration

Use this command to adjust and calibrate the size of each focus step.

Do not use this command if you are using a focus position encoder or an internal Z motor in your microscope.

For more information and procedures, see the Focus (Z- step) Calibration on page 28.

Final Magnification

The final magnification is the ratio of the size of the image divided by the size of the actual tissue. Think of it as the number of times larger the tissue must be made to make it the same size as the image. The final magnification describes the level of detail that is visible in an image. A number of factors contribute to the final magnification including the objective lens, the oculars, the monitor, and the video camera. The combined result of all of these factors is the final magnification.

Two calibration steps are necessary to determine the final magnification, monitor calibration, and lens calibration. The monitor calibration determines the size of the image in microns per pixel, while the lens calibration determines the physical size of a pixel.

To perform a final magnification calibration

The final magnification monitor calibration determines the size of a pixel on the computer's monitor. A calibration transparency or ruler marked in centimeters is needed to calibrate the monitor.

1. Click **Tools>Final Magnification**, and read the dialog box information.
2. Click **OK**.
3. Place the cursor over the anchor and drag to moves the entire grid. It is just as easy to shift the calibration transparency to match the transparency and the grid lines that run through the anchor.

4. Adjust the dashed grid until the transparency and the grid match.
    - To stretch the grid, move the cursor over a horizontal line or vertical line until the crosshair changes to a two headed arrow. This indicates that the cursor is directly over the line. Left click and drag the line until the grid matches the transparency. The horizontal and vertical lines can be adjusted at the same time by dragging a corner of the grid. It is recommended that the horizontal and vertical adjustments are made separately to arrive at the best possible results.

Any changes made to the grid can be removed by clicking the Undo button. The grid reverts back one change.

5. To complete the calibration process right click. There are 3 options.
    - The **Accept Final Magnification** option uses the final grid settings as the monitor calibration.
    - The **Undo Last grid Change** is the same as the **Undo** button.
    - The calibration changes are canceled if **Quit And Discard Changes To Final Magnification** is selected.

Click **Tools>Edit Lens** and choose a lens. The display of final magnification can be found in the **Edit Lens** dialog box, accessed by selecting **Tools>Edit Lens**, choose a lens and click **Edit**. Stereo Investigator displays the final magnification in the **Edit Lens Parameters** dialog box.

Shrinkage Correction

Section shrinkage is likely to be different along the X, Y and Z axes, particularly along the Z (depth) axis. The standard model for shrinkage assumes that the shrinkage is linear. This means that the shrinkage at the top of a section is the same as the shrinkage at the bottom of a section. In fact, it implies that the shrinkage occurs equally all along any axis of the tissue. Shrinkage correction is easy to apply when shrinkage is linear. The Z axis is multiplied by a factor to adjust the data for shrinkage.

Scaling Factors: For simplicity, assume that a section that was cut at 80µm shrinks to a thickness of 50µm. The final tissue is 5/8 the thickness of the original tissue. To apply a correction to restore the tracing to the original size, the data must be multiplied by 8/5. Enter a Z shrinkage correction of 1.6 (the decimal representation of 8/5). Multiply 50µm by 1.6 and the result is 80µm, which is the original thickness of the material. Data entered after applying Shrinkage Correction is not affected.

To apply shrinkage correction

1. Click **Tools>Shrinkage Correction.** Stereo Investigator displays the **Shrinkage Correction** dialog box.

2. Type a value for the Z axis or use the arrow buttons to make adjustments. If you want to see your changes as you make them, check **Dynamically Update. Also Adjust Images applies** the factor to images.
3. Click **OK** to apply the changes or click **Restore** to return to the original values.

If Shrinkage Correction is used without **Options>Display Preferences>View>Show Current Section Only** selected, all of the data in the file is modified to correct for shrinkage. If **Show Current Section Only** is selected, only the selected section is corrected.

It is recommended that, under normal conditions, Shrinkage Correction only be used in Stereo Investigator to adjust for Z unless sections are mounted upside down. Using **Adjust Scaling**, apply –1.0 to either X or Y and then in Shrinkage Correction apply –1.0 to Z to turn an existing tracing upside down so that it can be matched with a second section. When the upside down section has been traced, reapply –1.0 to these axes to return the tracing to the right side up position.

For more information, see Flip Sections Mounted Upside Down.

It is important to determine how much shrinkage each section, or series of sections, has undergone during preparation.

Adjust Scaling

Use **Adjust Scaling** to change the scaling factor for the X and Y axis.

To adjust scaling

1. Click **Tools>Adjust Scaling**. Stereo Investigator displays the **Adjust Scaling** dialog box.

2. Type a value for the X and Y axes or use the arrow buttons to make adjustments. If you want to see your changes as you make them, check **Dynamically Update. Also Adjust Images applies** the factor to images
3. Click **OK** to apply the changes or click **Restore** to return to the original values.

If Adjust Scaling is used without **Options>Display Preferences>View>Show Current Section Only** selected, all of the data in the file is modified to correct for shrinkage. If **Show Current Section Only** is selected, only the selected section is corrected.

Match

Match is useful for aligning serial sections or for aligning data with a tissue section that has been placed on the microscope again. **Match** changes the alignment of all existing sections (hidden and visible) to match the current section.

Realigning a tracing with the section it was traced from poses a problem if the section is oriented differently on the microscope. **Match** provides a two, three, four, or up to 99, point pair matching procedure for alignment. The **Match** algorithm performs an optimal rotation and translation of the tracing overlay to align the tracing with the image of the new section. The match between consecutive points chosen by the user is made using a least square error technique using the XY coordinates of the chosen points.

To match

1. Click **Tools>Match.** Stereo Investigator displays the **Match** dialog box.

2. Select or type the number of point pairs to use for matching the tracing with the section, and click **OK.**
3. Pick a point on the overlay to match to the image. Then pick the corresponding point on the image. Repeat this for subsequent point pairs. This may require moving the stage to find the next pair of points to match. Use the **Move>Joy Track,** or the arrow buttons on the status bar to find point pairs which lie outside of the current field-of-view.
4. When all matched pairs have been selected, Stereo Investigator translates the tracing and rotates it to the optimum match.

Things to remember

- Match is much faster than **Rotate Tracing** and is the easiest method to obtain a quick, good fit between the tracing and the image.
- Match operates on both visible and hidden data in sections that are not suppressed. Suppressed sections, whether they are visible as gray or invisible, are not affected.
- It is possible to use the **Where Is** operation and the **Go To** operation at any time to find and place any **Match** fiducial points that are not in the original field-of-view. The fiducial points placed in the **Match** operation are visible in **Where Is.**

Right Button Options

Undo

The last point of a pair can be deleted (in order to place it somewhere else) by selecting this option from the right mouse button menu.

Accept as Is

If you have chosen a large number of point pairs, you can select this option before all of them have been placed to complete the **Match** operation.

End Match

The match procedure can be canceled by selecting **End Match** from the right mouse button menu. The tracing is not moved from its original location.

Match Section

Match Section works the same as the **Tools>Match** with the exception that it only re-aligns one section and not the entire stack. Use **Tools>Match** to align each section as you go while tracing serial sections. Use Tools>Match Section... to trace all of the sections first, then do the alignment. Using Match Section allows you to align each section with the one immediately adjacent to it, moving the new section rather than the whole stack.

Match Section will not work if all sections are currently displayed. To use **Match Section**, select **Show Current Section Only.** If the other sections are required for the alignment, use **Show Suppressed as Gray** to see their locations.

Match Section only acts on the active section as defined in the **Serial Section Manager**.

To match

1. Click **Tools>Match Section.** Stereo Investigator displays the **Match** dialog box.

2. Select or type the number of point pairs to use for matching the tracing with the section, and click **OK.**
3. Pick a point on the overlay to match to the image. Then pick the corresponding point on the image. Repeat this for subsequent point pairs. This may require moving the stage to find the next pair of points to match. Use the **Move>Joy Track,** or the arrow buttons on the status bar to find point pairs which lie outside of the current field-of-view.
4. When all matched pairs have been selected, Stereo Investigator translates the tracing and rotates it to the optimum match.

Things to remember

- Match is much faster than **Rotate Tracing** and is the easiest method to obtain a quick, good fit between the tracing and the image.
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The last point of a pair can be deleted (in order to place it somewhere else) by selecting this option from the right mouse button menu.

Accept as Is

If you have chosen a large number of point pairs, you can select this option before all of them have been placed to complete the **Match Section** operation.

End Match

The match procedure can be canceled by selecting **End Match** from the right mouse button menu. The tracing is not moved from its original location.

Rotate Tracing

Rotate Tracing rotates selected objects clockwise or counterclockwise about the reference point. This feature is useful for graphically aligning serial sections or for aligning data with a tissue section that has been placed on the microscope again. The tracing dynamically rotates as you press the directional arrows, letting you see the progress of the rotation.

- If **Options>Display Preferences>View>Show Current Section Only** is enabled, Stereo Investigator rotates only the data belonging to the current section.
- Unlike other editing modes, even when **Show Current Section Only** and **Show Suppressed As Gray** are enabled, the suppressed section data shown in gray is not rotated. This allows aligning of the tracing of the current section with previous section data.
- Hidden objects in the current section, or in any section if **Show Current Section Only** is not enabled, are rotated along with the visible objects.

Hide Tracing

Hides the tracing. To see the tracing, click **Hide Tracing** again.

Acquisition Menu

Live Image

The **Live Image** command displays a real time video image. For systems configured with a motorized shutter, **Live Image** causes the fluorescence shutter to open and remain open for as long as **Live Image** is selected. When **Live Image** is deselected , the shutter closes.

Display Acquired Image

All acquired images are displayed. This feature is grayed out (disabled) if there are no acquired images. An acquired image can be one acquired from the frame grabber via **Imaging>Acquire Image**, or one obtained by reading a previously captured image via **File>Image Open**. This tool does not display a grabbed image, only the ones that have been acquired.

Images are embedded in 3D-space. This means the images are placed at the (X, Y, Z) position where they were acquired and also at the size they were acquired. If you have trouble finding an image, right click in the **Macro View** window and select **Display Acquired Images** to see the location of all images and the current field-of-view. Images acquired at a low power appear larger than images acquired at a high power. Images maintain their relationship even if a new reference point is placed. Unlike tracing data, images are not discarded when you create a new file.

To restore **Live** mode, deselect **Display Acquired Image.**

Use the **Image Organizer** to discard unwanted images. If the images overlap, the layering order of images is also controlled using the **Image Organizer**.

Display Live and Acquired Image

Displays both the live and the acquired images, blending them together. This feature is grayed out (disabled) if there are no acquired images and if no live image is available. An acquired image can be one acquired from the frame grabber via **Imaging>Acquire Image**, or one obtained by reading a previously captured image via **File>Image Open**.

Images are imbedded in 3-space. This means the images are placed at the (X, Y, Z) position where they were acquired and also at the size they were acquired. If you have trouble finding an image, right click in the **Macro View** window and select **Display Acquired Images** to see the location of all images and the current field-of-view.

Images acquired at a low power appear larger than images acquired at a high power. Images maintain their relationship even if a new reference point is placed. Unlike tracing data, images are not discarded when **File>New** is performed.

Use the **Image Organizer** to discard unwanted images. If the images overlap, the layering order of images is also controlled using the **Image Organizer**.

Adjust Video Input

When using a video camera, **Adjust Video Input** lets you adjust the input from the camera. The camera settings themselves are on the camera controller. When using a digital camera, **Adjust Video Input** acts to adjust the settings of the camera as well as the inputs.

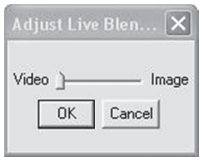
Adjust input parameters, such as brightness, contrast, etc. For this function to operate, your system must have a frame grabber board supported by MBF Bioscience.

Three buttons appear at the bottom of the dialog box. Pressing **OK** makes the changes permanent. Pressing **Cancel** causes any changes to be ignored. Pressing **Defaults** restores the default settings appropriate to the board and signal format being used.

If you are using the MicroFire or MacroFire camera, the dialog box is the same as in the Optronics Picture Frame software. Please refer to the camera manual for settings and information.

Video Blend

Displays the **Adjust Live Blend** dialog box,



which you use to adjust the blending of the live image with the static image on screen.

Move the slider to the left to add more of the video image; move the slider to the right to add more of the static image.

Use External Image Sources

You can place external images in Stereo Investigator.

External Image Source Setup

The **External Image Source Setup** dialog can be used to inform the system that you plan on loading images from an external source while controlling the stage. An external image source can include things like a laser confocal microscope that Stereo Investigator may not be configured to control. If images are loaded at a time when this application is controlling the stage then it may be necessary to request more input from the user in order to assure the image is loaded into the world coordinate system correctly. An example of requesting more information is the **Image Placement** section that is added to the **Image Scaling** dialog used when opening an image.

Using the **External Image Source Setup** to inform the system of your intentions will help the system do a better job at preselecting the options you are likely to want and in some cases it can avoid having to request extra user input. If you check the checkbox in the setup dialog then you should also enter the pixel resolution of the images you will be loading. If the system does not have a camera configured then the image size in this dialog will be used in the calculation of the current view of the world coordinate system. The current view of the world coordinate system is what determines the placement and magnification of the elements of your data file (tracing and any previously acquired images) on the main display window. If the system does have a camera configured you can use **Acquisition>Use External Image Source** to tell the system to use this setting for calculating the current view of the world coordinate system. If your system is configured to drive a confocal microscope then there are instances where this configuration will happen automatically without ever bringing up this dialog.

Use External Image Source

If you plan on loading images from an external source while you are controlling the stage and you have used the **External Image Source Setup** dialog under the **Options** menu to tell the system how big the external image source is (in pixels) then you can now use **Acquisition>Use External Image Source** to ask the system to use the External Image Source image size to calculate the current view of the World Coordinate system. This specifies that you are NOT using the image size from the camera so turning this on will turn the live image off. If you turn live back on, the Use External Image Source will be shut off. This also assumes that you want to link the placement of the world coordinate system to the stage position so the display of acquired bitmaps will be turned off unless you are using **Move>Synchronize Stage and Images** This is so that you will maintain control of the stage. If you turn **Use External Image Source** on and then set the current lens to one that is appropriate for the external image source then the view of the world coordinate system will be calculated so that the elements of your data file (tracing, markers, any previously acquired images) will be displayed in the appropriate scaling and position for the new image to

be read into the upper left corner of the main display window. It is important that you read the section on the Prepare for External Image Source menu option because you may want to get in the practice of using that menu option instead of this one to turn on this feature. The difference between the two entries is explained there.

If you do not have a camera configured in your system, then this option is not available because the system will already be using whatever is defined in the External Image Source Setup. If your system is configured to drive a confocal microscope then this menu entry may be replaced by the **User Laser for Acquires** entry.

Use Laser for Acquires

This command may be enabled when the system is configured to use a confocal microscope while also being configured for a digital camera. Checking this option specifies that the image size of the laser should be used instead of the current camera live image size when calculating how to place the world coordinate system onto the main display window (the world coordinate view). This is the calculation that determines the placement and magnification of the elements of your data file (tracing, markers, previously acquired images). This setting only has an impact when the current view of the world coordinate system is tied to the current stage position. he two most common cases of that are when the camera is live or when no images are being displayed (**Display Acquired Image** is off). Checking this menu option is the same as selecting the **Use Laser** option in the generic **Camera Settings** dialog (**Acquisition>Video Tool Panel->Camera Settings**). This menu entry may replace the **Use External Image Source** menu entry because it is used to accomplish the same thing but in this case you know the “external image source” is a laser confocal microscope.

There are two hardware configurations that allow this option to be selected. Both configurations require that the system be configured to drive a confocal microscope. Both configurations have to also have a camera configured. One configuration has a “camera” configured that can get images from either the laser or a digital camera controlled by the confocal scope application. he ZEN camera is an example of this. In this case, **Use Laser for Acquires** is like switching cameras. When the laser option is used, the size of the "live" image from the camera changes to match what is delivered by the laser. When the camera is put in live mode, you will get images from the laser. The second configuration has a camera object that can only get images from a real digital camera and not the laser.

Note this configuration is more complicated to use and in some cases (ZEN integration) can and should be avoided by selecting the **Add Option to Get From Laser** option in the **Settings** tab of **Camera Setup** dialog box.)

Checking the **User Laser for Acquires** here is exactly the same as selecting **Use External Image Source**. It will turn live off and use the image size configured in the **External Image Source Setup**. It will also check to see if it needs to turn off the display of acquired images in order to get control of the stage. You might not ever see the **External Image Source Setup** dialog because the configuration is done automatically for you since it is known that this is a source of images external to the camera and the size of the external image source can be obtained from the confocal scope application. If your system has this second configuration then you will also see an **Enable Laser Acquires** menu option.

Enable Laser Acquires

This command is available for systems configured to drive a confocal microscope but are configured to use a camera not controlled by the confocal microscope and have not added the option to get images from the

laser. In this case you cannot get a live laser image. This configuration uses the External Image Source feature to maintain registration between the images from the camera and images from the laser. In this case the External Image Source is a laser so the **Use External Image Source** command is replaced by the **Use Laser for Acquires** command and the Prepare for External Image Source menu option is replaced by the **Enable Laser Acquires** menu option. Choosing **Enable Laser Acquires** is the same as toggling **Use Laser for Acquires** from unchecked to checked. It is useful when you have acquired an image from the laser and have lost the ability to drive the stage because your acquired image is now being displayed. For a full explanation of this feature, see Use External Image Source and Prepare for External Image Source.

Acquire Image

his operation transfers a bitmapped image of the current video image into memory. The bitmapped image is known as an *acquired image*. When a video image is acquired, it is displayed until **Live Image** is again selected. All acquired images are saved in their "real" location, as related to the reference point.

Acquire Multichannel Image

Starts an acquire of an image where the color channels are split. Stereo Investigator acquires the Red, Green, and Blue channels separately. See Automating Your Acquires starting on page 59 for information on multichannel acquires.

Acquire Image Stack

This command automatically captures the current field-of-view at different focal depths into multiple bitmapped images. The collection of these images is called an *image stack.*

Select **Imaging>Acquire Image Stack** to display the **Image Stack Acquisition** dialog box, which contains these command options.

- **Distance below top of section for first image:** After you have manually focused at the top of the section, the software focuses down this distance before acquiring the first image of the stack. You can keep this value 0.0 if you would like the image at the top of the stack to be the first image in the stack.
- **Distance between images:** Determines how far the software focuses down into the section before acquiring each new image.
- **Thickness of image stack:** Determines how far down the software focuses from the original focal depth. If you do not know the thickness of the image stack, you can determine it by using the Focus Position Meter (**Options>Display Preferences>Accessories** and check the **Focus Position Meter**) to compare the height of the first focal plane in the section to that of the last.

The lens could break the slide if the thickness is set greater than the thickness of the tissue!

- **Time Delay:** Allows for a designated amount of time for the system to "settle" before acquiring the next image. This allows for any vibrations due to stage motion to die down.
- **Refocus at top of section before acquiring image stack:** If this option is not selected, the image stack acquisition starts from the current Z position. If you are not currently focused at the top of the image stack, selecting this option allows manual focusing prior to the acquisition.

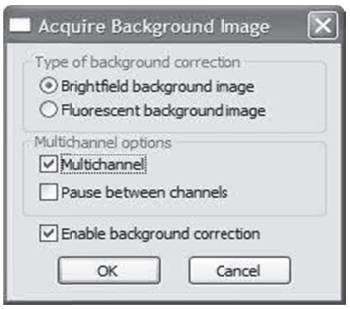
- **File name base:** Each image stack file is saved directly into the folder on your computer's hard drive, so a file name must be designated before the acquisition can begin. Do not give this file name an extension, the software assigns an extension automatically.

Acquire Virtual Tissue

Acquires a virtual slice from the live image. For more information and procedures, see The Virtual Tissue Module on page 103.

Acquire Background Image

Displays the **Acquire Background Image** dialog box,

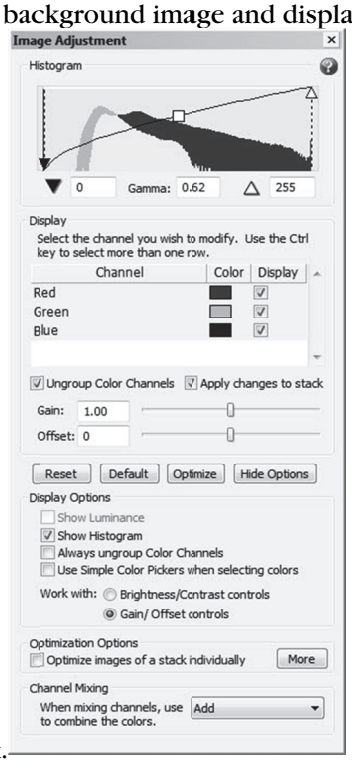


which you use to set options and then acquire a background image.

To acquire a background image

1. Make sure **Live Image** is selected in the Image menu.
2. Click **Imaging>Acquire Background Image**. Stereo Investigator displays the **Acquire Background Image** dialog box.
3. Select the options you wish to use.

Click **OK. Stereo Investigator** acquires the background image and displays the **Image Display Adjustment** dialog box.



4. Make any adjustments to the image. For information on the **Image Display Adjustment** dialog box, its options, and uses, see Image Display Adjustment.

Acquire Background Image Options

Type of Background Correction

- **Brightfield background image** or **Fluorescent background image:** Choose which type of image for background correction.

Multichannel options

- **Multichannel:** Choose if you are acquiring a multichannel background image.
- **Pause between channels:** Available if the **Multichannel** option is selected. Select this option if you want Stereo Investigator to alert you when a channel has been acquired, so that you can make any changes or adjustments to the slide.

Enable background correction

Select this option to enable background correction, which sets the background levels based on the background image.

Acquire Setup

The **Acquire Setup** command lets you automate some of the repetitive tasks associated with the **Acquire Image, Acquire Multichannel Image, Acquire Virtual Slice,** and **Acquire Image Stack** operations. For more information and instruction, please see the Automating Your Acquires on page 59.

Acquire SRS Image Series and Acquire SRS Image Stack Series

There are at least two ways to run a Fractionator or Optical Fractionator on a FLUOVIEW system. One way is to do an Acquire SRS Image Series or an Acquire SRS Image Stack Series (and then run the Fractionator or Optical Fractionator on the acquire. Another way is run a Fractionator or Optical Fractionator as you would on a standard bright-field microscope but use the FLUOVIEW application to focus, and use Stereo Investigator 's Acquire Image or Acquire Image Stack operations to acquire images at the site when it is time to count. The setup for running an (Optical) Fractionator and an Acquire SRS Image (Stack) Series is the same.

Setup

1. Set the Z stage to FLUOVIEW (if it is not already set at FLUOVIEW), and move the stage until you are over the upper left corner of the region of interest.
2. Click a reference point.
3. Click **Move>Synchronize Stage and Images** or the **Synchronize Stage And Images** button.

   If you don't synchronize the stage and images and you have an acquired image displayed, the hardware stage will not be driven.

4. Make sure you have selected the correct lens.
5. If you are planning to work with multiple sections, click **Tools>Serial Section Manager** and define a section.
   - In the **Serial Section Manager** dialog box, click **New Section**.
   - Type the values for the section and click **OK** to define the section, and then click **Close** to close the **Serial Section Manager**.

   If you do not want to define any sections, it is possible to proceed without this step but you will be notified that the **Serial Section Manager** is not being used when you start an **Acquire SRS Image (Stack) Series, Fractionator,** or **Optical Fractionator**. You can ignore this message.

6. If you have the Virtual Slice module for Stereo Investigator, perform an Acquire Virtual Slice and then go to step 11. To perform the **Virtual Slice**, select a Grid Scan and specify enough X and Y fields to cover your region of interest, no Virtual Slice File, no Seam Alignment, no Seam Blending, no 3D. When you press **OK**, Stereo Investigator displays a grid of possible focus locations. You can right click to either specify sites you want to focus at, or to start the scan. If you guess wrong on how many X and Y fields, you might have to try again. When you succeed, go to step 11.

If you don't have the Virtual Slice module for Stereo Investigator, then use the following steps to accomplish the same result. In Stereo Investigator, click **Imaging>Acquire Image**.

Choose the source for scaling information, and choose **Confocal Scan.**

7. Move to the right by clicking the right arrow button or using **Joy Track** mode with the joy stick.

   You can adjust the amount of movement associated with one button click by changing the setting on the of **Options>General Preferences>Movement** tab. You want to move just enough so that the next acquired image will be next to the one you just acquired.

8. Repeat steps 6 and 7 until you have acquired enough images to span your region of interest from left to right.
9. Move down using the down arrow button.
10. Repeat steps 8 and 9 until you have acquired enough images to span your region of interest from top to bottom.
11. Trace your region of interest using the images you have just acquired.
12. If you have not done so, click **Probes>Define Counting Frame**. Both the size and the position are important here. You want to position it so that if you acquire an image, the counting frame will be over that image.
13. Click **Probes>Preview SRS Layout**. If you want Stereo Investigator to estimate a grid size to generate a specific number of sampling sites, then type the desired sampling sites and click **Estimate Grid Size**, then click **OK**. When you have finished looking at the grid, right click the mouse and choose **End Preview Mode**.
14. If you want to do the acquires up front, click **Acquisition>Acquire SRS Image Series** or **Acquisition>Acquire SRS Image Stack Series**. Directions for running the **Fractionator** or **Optical Fractionator** from the acquire are listed in the next sections. If you want to run a **Fractionator** or an **Optical Fractionator** in the more traditional way, you can choose **Probes>Fractionator** or **Probes>Optical Fractionator**. Directions for this are also in a section below.

Acquire SRS Image Series (Single Image each site)

1. You will be acquiring a single image at each sampling site. If you have more than one contour in your tracing, you are presented with a view of all the contours. Click on the contour surrounding your region of interest. If you only have one contour, it is already selected.
2. The **Acquire SRS Image Series** dialog box lets you change the Grid Size.
   - **None:** performs a focus scan will be done at the very first site.
   - **Pause for Manual Focus:** performs a focus scan at every site.
   - **Autofocus:** uses the built-in autofocus property.

   Set the **Path** and **File Base Name** and click **OK**.
3. If you have multiple contours in your tracing, you are asked to pick another contour to acquire from.
4. When you have finished adding contours to your acquire, Stereo Investigator drives the stage to the first site and you displays a message box stating: A focus scan will now be started. Stop the scan in the FLUOVIEW application when in focus. Click **OK**.
5. Stereo Investigator should now start a focus scan in the FLUOVIEW application. Adjust the Z Position in the FLUOVIEW application until the slide is in focus. This can be done from the **Z Stage** tab in the lower left corner of the FLUOVIEW application. Now press the **Stop Scan** button at the upper left corner of the FLUOVIEW application to tell it that it is now in focus.

6. Stereo Investigator asks FLUOVIEW to acquire an image, and then reads the image into Stereo Investigator. Stereo Investigator displays the **Image Scaling** dialog box. You can change the scaling information for this image. The scaling information should be correct as it is; click **OK**.
7. If you selected the **Focus At Each Site** option, Stereo Investigator moves to the next site and starts a focus scan. Stereo Investigator asks you to stop the scan when in focus just as you it did at the previous site. This continues for each of the sites. If you selected the **Focus At Each Site** option, *but* you selected multiple contours, Stereo Investigator will still ask you to focus at the first site of each contour. If you did not select the **Focus At Each Site** option, Stereo Investigator drives the stage to each of the sites and acquires an image at each site.
8. Once all the images have been acquired, save your file. You can save your file, exit Stereo Investigator and FLUOVIEW and come back at a later time to run the Fractionator. If you do exit Stereo Investigator, all you have to do when you restart Stereo Investigator is to load your data file again.
9. Click the **Synchronize Stage and Image** button again. We don't need to be driving the stage at this point, but it is okay to drive it if you prefer.
10. Click **Probes>Select Acquired SRS Series**.
11. There should be one entry in the dialog that represents the one SRS Series you just acquired. Select that entry and press **OK**.
12. You should now see the counting frame and the image that was acquired at that site. Now proceed as if you were running a regular **Fractionator**. See for more information.

Acquire SRS Image Stack Series (Stack at each site)

1. You will be acquiring a stack of images at each sampling site. If you have more than one contour in your tracing, you are presented with a view of all the contours. Click on the contour surrounding your region of interest. If you only have one contour, it is already selected.
2. The **Acquire SRS Image Stack Series** dialog box lets you change the **Grid Size**. Some of the information for this dialog will automatically be filled in using the currently defined section if there is one.
    - If you check **Include guard zone images**, then images will be taken of the entire section.
    - If you don't check **Include guard zone images**, then images are taken of the **3D Counting Frame**.
    - If you check **Manual focus to top of section at each site**, then a focus scan runs at each site.
    - Select **Optical Fractionator** in the drop down list box. Set the **Path** and **File Base Name** and click **OK**.
3. If you have multiple contours in your tracing, Stereo Investigator lets you pick another contour to acquire from.
4. When you finish adding contours to your acquire, Stereo Investigator asks if you want to perform the top of section settings up front. If you answer **Yes**, then all the top of section focus operations happen before any of the acquires. If you answer **No**, then acquires happen after each focus operation.

5. Stereo Investigator drives the stage to the first site and you displays a message box stating: A focus scan will now be started. Stop the scan in the FLUOVIEW application when in focus. Click **OK**.
6. Stereo Investigator should now start a focus scan in the FLUOVIEW application. Adjust the Z Position in the FLUOVIEW application until the slide is in focus. This can be done from the **Z Stage** tab in the lower left corner of the FLUOVIEW application. Now press the **Stop Scan** button at the upper left corner of the FLUOVIEW application to tell it that it is now in focus.
7. If you answered **Yes** to performing the top of section settings up front, then steps 5 and 6 repeat for other sites until all the top of section focus operations are finished. You then get a dialog that tells you that the acquire process is ready to begin. Other than that last dialog that tells you the acquire process is ready to being, all the operations are the same as if you had answered **No** to performing the top of section settings up front. The only difference is the order in which those operations happen. In an effort to simplify these directions, the rest of the steps will assume you answered **No** to performing the top of section settings up front.
8. Stereo Investigator asks FLUOVIEW to acquire an image stack, and then reads the image stack into Stereo Investigator. Stereo Investigator displays the **Image Scaling** dialog box. You can change the scaling information for this image. The scaling information should be correct as it is; click **OK**.
9. If you selected the **Manual focus to top of section at each site** option, Stereo Investigator moves to the next site and starts a focus scan and asks you to stop the scan when in focus just as you were asked to at the previous site. This continues for each of the sites. If you did NOT select the M**anual focus to top of section at each site** option, Stereo Investigator drives the stage to each of the sites and acquires an image stack at each site. You are also asked to focus at the first site for each contour.
10. Once all the images have been acquired, save your file. You can save your file, exit Stereo Investigator and FLUOVIEW and come back at a later time to run the **Optical Fractionator**. If you do exit Stereo Investigator, all you have to do when you restart Stereo Investigator is to load your data file again.
11. Click the **Synchronize Stage and Image** button again. We don't need to be driving the stage at this point, but it is okay to drive it if you prefer.
12. Click **Probes>Select Acquired SRS Series**.
13. There should be an entry in the dialog that represents the SRS Series you just acquired. If you selected more than one contour, there are multiple entries in the dialog where each entry is associated with one contour. Select an entry to run the estimator on. **Optical Fractionator** should already be selected with the correct counting frame specifications. Click **OK** to start **Optical Fractionator**.
14. You should now see the counting frame and the images that were acquired at that site. Now proceed as if you were running a regular **Optical Fractionator** but use the PgUp and PgDn keys (or mouse wheel) to focus up and down through the stacks.
15. If you did not use the M**anual focus to top of section at each site** option, you may find that the top of section may not be correct from site to site. To reset the top of section at a site, right click the mouse and select **Reset Top of Section**. Stereo Investigator tells you to: **Focus at the top of the section and then click on OK**. You can then use the mouse wheel or PgUp and PgDn keys to move up and down in the stack until you are at the true top of section at which time you should click **OK** to continue.

Traditional Fractionator or Optical Fractionator

1. If you are performing an **Optical Fractionator**, select **Manual focus**. For both **Fractionator** and **Optical Fractionator** you should enter your options and click **OK**.
2. You may be asked to focus at a site. To focus, start a Focus scan in FLUOVIEW and use the arrow controls on the **Z Stage** tab of the **Acquire** page in FLUOVIEW to adjust Z position to bring the image into focus and then stop the focus scan. When it is in focus and you have stopped the focus scan, click **OK** in the focus dialog in Stereo Investigator. If you are running an **Optical Fractionator**, you may be asked to focus at the top of the section and the bottom of the section so you will do this focus procedure twice.

Make sure you have stopped the focus scan before continuing.

3. Stereo Investigator asks you to select a marker to count cells. At this point you would normally be looking at a live image but because this is a FLUOVIEW system, you will need to acquire an image or, in the case of an Optical Fractionator, acquire an image stack. Click **Acquisition>Acquire Image** or **Acquisition>Acquire Stack**.
4. Now use markers to count your cells. You can use PgUp and PgDn to move through your image stack.
5. Make sure that the **Synchronize Stage And Images** button is still depressed. The stage will not move if acquired images are being displayed and the button is not pressed.
6. When you are done counting at that site, right click and select **Next Scan Site**.
7. Repeat steps 3 through 6 until all sites have been visited.

There are some advantages and disadvantages for each method of running a **Fractionator** or **Optical Fractionator**. If you use **Acquire SRS Image Series** or **Acquire SRS Image Stack Series**, it should be possible to repeat the acquire process for each of the sections and then do all the counting at a later date on a separate machine so as to free up the microscope. If you acquire images on the fly while doing the **Fractionator** or **Optical Fractionator**, you may discover potential problems a little earlier in the process. Either method should work. The second method currently has a greater risk of fouling the communications between FLUOVIEW and Stereo Investigator because you are starting and stopping focus operations from the FLUOVIEW application window. If you start a focus scan from FLUOVIEW and forget to stop it, and then Stereo Investigator asks FLUOVIEW to acquire an image or an image stack, the two applications are left in a bad way.

Acquire SRS Image Workflow

Starts the Workflows for these commands. Complete help is available within the Workflows.

Set Brightfield to Background Image

When selected, uses the acquired image in a multi-white correction.

Set Fluorescent to Background Image

When selected, averages the colors of a selected dark area.

Display Background Image

Displays the image acquired for background correction.

Enable Background Correction

Sets the background levels based on the background image.

Video Tool Panels

Use to display and edit the Camera Exposure, Multichannel, and Video Histogram dialog boxes.

Camera Exposure

Toggles the display of the **Camera Settings** dialog box.

You can increase the exposure by moving the slider to the right; decrease exposure by sliding to the left. You can also use the spinner buttons (arrows) to decrease or increase exposure .

Click the **Automatic** checkbox to set an automatic exposure, which overrides any **Exposure** setting you set.

Use the **Gain** slider to increase or decrease the signal power from the camera. Remember that as you increase gain, more noise is introduced into the image. Note that this is not present for cameras that do not support gain.

The **Binning** option reduces the number of pixels coming from the camera. A value of '1' means no binning, a value of '2' means reduce 1/2 the pixels.

The **Set to Mono/Set to Color** button switches between color and monochrome for cameras capable of this feature.

Click More to display more options specific to your camera.

Multichannel

Toggles the display of the **Video Multichannel** dialog box.

You use the **Multichannel** dialog box when using the Image Stack module and acquiring images by color channel. You can set specific actions (depending on hardware installed) to occur before, during, and after a channel acquire. For more information on setting channel options, see the Acquire Setup discussion.

Select **Remember Video Settings** if you have made changes to the video settings and you want to use them again.

Video Histogram

Toggles the display of the **Video Histogram** dialog box. The **Video Histogram** displays a graphical representation of the visual brightness and contrast of the current image. Absolute black (0%) is the lower-left corner, while absolute white (100%) is the upper-right corner.

Note the **Lum,R,G,B** buttons. **Lum** is the default and displays luminance, which is a weighted sum of red, green, and blue. **R,G,B** change the display to be only Red, Green, or Blue channels. You can make adjustments to the display by dragging the handles within the histogram. Click Optimize to display an

optimized (best case) view of your image. Click Reset to discard any changes you made and return to the original display.

Use **Clip Detect** to find and display any clipped pixel (max intensity value) with the Key color. In this way you can easily spot which areas are overexposed and make adjustments. You can set **Clip Detect** values on your camera's **Setup Tab** under **Options>Video Setup**.

Image Menu

Image Adjustment

The **Image Adjustment** command lets you make changes to the way Stereo Investigator displays the current image, with the **Image Display Adjustment** panel.

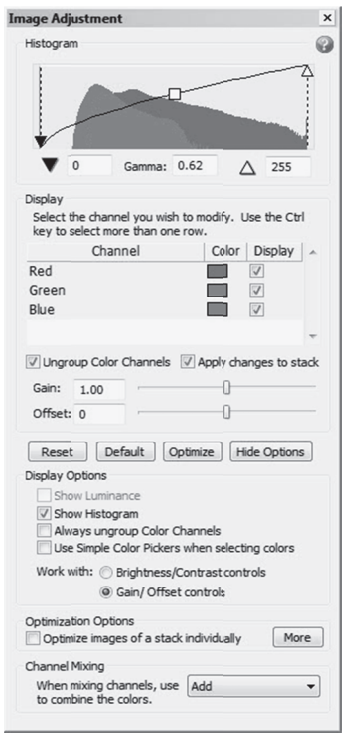


Image Display Adjustment controls and options

Histogram

Displays a graphical representation of the tonal values in the image. It has the following controls. which you can grab with the mouse and move or change values in the text boxes:

- **Black point:** The point at which solid black occurs.
- **Gamma:** Controls the overall brightness of an image.
- **White point:** The point at which solid white occurs.

Display

Displays the available channels. If **Ungroup Color Channels** is selected, each channel is listed and may be modified. All channels are initially displayed. To hide a channel, clear the check box next to it.

Select **Apply changes to stack** to have the changes applied to each image in the image stack.

- **Brightness** : The luminance of a pixel; the total amount of light in the color.
- **Contrast:** The difference in brightness between the light and dark areas.

These controls may be displayed as **Gain** and **Offset**, if you chose that option under **Display Options.**

Commands

- **Reset** will reset the image to its original settings up to the last save.
- Default sets the **Black Point** to 0 and the **White Point** to the highest value available for the image's bit rate. For an 8-bit image, the highest value is 255. For a 12-bit image, it is 4095.
- **Optimize** moves the **Black Point** to 0 and the **White Point** to the brightest value available for a pixel. You can apply this command to one or more selected channels at a time.
- **Show/Hide Options:** Shows or hide the display options for this tool panel. These options are:
    - **Show Luminance:** Displays the luminance in the histogram display. This option is only available if **Ungroup Color Channels** is selected.
    - **Show Histogram:** Displays the histogram.
    - **Always ungroup Color Channels:** Displays the color channels as ungrouped in the **Display** listing.
    - **Use Simple Color Pickers when Selecting Colors:** Determines which color Stereo Investigator displays.
    - **Work with Brightness/Contrast controls** or **Gain/Offset controls:** Determines which of these controls to display in the **Display** section.

Channel Options

You can use any of these actions to combine channels:

- **Add** will add the values of the selected channels together. The resulting value is never higher than the highest value available, 255 for 8-bit images, 4095 for 12-bit images, etc. For example, if the red channel value is 100, the green channel value is 20, and the blue channel value is 200, the result is 255.
- **Max** uses the maximum value of the channels. For example, if the red channel is 60, the green channel is 230, and the blue channel is 225, the resulting value is 230.
- **Average** displays the average value of all channels.
- **Or** will perform an "OR" operation on the channels.

Image Processing

Use the **Image Processing** command (formerly **Image Effects**) to change the characteristics of an image. The two main reasons for modifying images is to make the images easier for the human eye to interpret and to enable automated processing. For example, the automated tracing and particle counting functions require a black and white (Kodalithed) image, as free from background noise as possible. Preparation of these images requires the use of **Image Processing** tools.

More on Image Processing

To use **Image Processing**, click **Imaging>Image Processing**, or click the **Image Processing** button. The **Image Processing** menu provides you with the ability to perform various image processing operations on an acquired image. The range of image effects available depends on the type of image that is acquired.

There are 4 general categories for images:

- **True Color Image:** This is the normal image. A true color image is composed of pixels that are varying amounts of red, green, and blue. Each of the three primary colors has a value from 0 to 255. This is the dynamic range of each color component. All images acquired from a color camera are true color images.

Paletted images: A paletted image is usually a compressed image. The difference between a true color image and a paletted image is that the paletted image only contains a few hundred colors at most, with each color coming from a limited list called a palette. Although Stereo Investigator supports paletted images, it is recommended that images are not converted to the paletted form. The transform from a true color image to a paletted image loses information

- **Grayscale images:** A grayscale image is similar to a true color, but the pixels have only a single component: a brightness value that ranges from 0 to 255.
- **Monochrome images:** A monochrome image is just black and white. The dynamic range is 0 and 1. A 0 is black and a 1 is white. For purposes of comparison to other images these values are changed to 0 and 255, with the value 255 being white. Monochrome images are very important in image processing. The image is seen as two distinct regions. One region, usually the black is considered the foreground. These are the objects that are of interest. The other region is considered to be the background. This is the portion of the image that is not of interest. Splitting the image into foreground and background components is called image segmentation.

The word *monochrome* is often used to classify cameras that actually capture grayscale images. A monochrome image contains only black and white pixels, with no shades of intermediate gray.

Using Image Processing

The **Image Processing** dialog box displays the settings and options for the last image effect you used. Each effect has different options. However, the **Image Processing** dialog box always contains an **Apply** and **Undo** buttons, a **Preview** and **Record** checkboxes, a **Favorites** button you use to record macros and define your favorite image effects, an **Effects** button used to choose which effects to use, and a **Close** button.

To use Image Processing

1. Click **Imaging>Image Processing.**

2. Select **Preview**. Stereo Investigator draws a marquee on the image. This is the preview area. You can move or resize this area with the mouse. Double click in the preview area to enlarge it to the size of the image. Double click again to restore the preview area to its previous size. Before applying an effect test the effect on the entire image by double clicking inside of the preview area.
3. Click the **Effects** button, and choose and an image effect and its option. For example, choose **Sharpen**, then choose **Sharpen, Sharpen Gentle,** or **Unsharpen**.
4. Choose or change any options for the effect. Any changes show up in the preview area.
5. When satisfied, click the **Apply** button. You can also click the **Undo** button to reverse the effect.
6. Click **Close** when complete.

Recording Image Processing Effects

Sometimes you need to apply an effect or set of effects to a group of images that aren't all loaded. You can record a macro to work more efficiently.

To record a macro of image effects

1. In the **Image Processing** dialog box, select the **Record** check box.
2. Select and apply image effects.
3. When you are done, click the **Favorites** button and choose **Macro>Save Macro as.**
4. In the **Save Current Macro As** dialog box, type a name for the macro and click **OK**.
   The macro is now available from the **Favorites** button.

Adding Favorite Image Processing Effects

You can define a set of favorite, or often used, image effects that you can access with the **Favorites** button.

To define a set of favorite image effects

1. Click the **Favorites** button and then click **Define Favorites**.
2. In the **Define Favorites** dialog box, choose an effect in the left column and click **Add**.
3. Continue until you have selected the image effects you want as favorites.
4. Click **OK**. Your favorites are now available through the **Favorites** button.

Undo Image Processing

Undoes any **Image Processing** commands performed up to the last save.

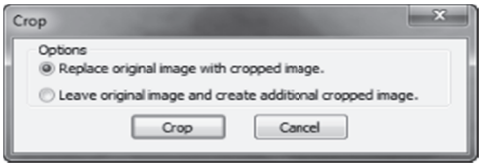
Crop Image

Lets you crop the image on screen. You are given the option to replace the original image in memory with the cropped image or to leave the original image in memory and create an additional cropped image. Stereo Investigator adds the cropped image to the list in Image Organizer.

You cannot undo a cropped image.

To crop an image

1. Click **Image>Crop Image**. Stereo Investigator draws a marquee around the cropping area and displays the **Crop** dialog box.



2. Move the mouse pointer over the image. When it reaches the crop marquee the mouse pointer changes to a double-headed arrow.
3. Drag the marquee to adjust the size of the cropped image.
4. Select an option and click **Crop**.

Deconvolve Image

Starts the Deconvolution module. Deconvolution reverses the optical distortion that takes place in an optical microscope to create clearer images. It can sharpen images that suffer from fast motion or jiggles during capturing or images that have some type of noise introduced into the signal.

For information and instructions, see The Deconvolution Module on page 83.

Invert Displayed Image

Inverts the colors the currently displayed image. This can be useful when trying to see subtle structures. If you are viewing a image stack, **Invert Displayed Image** inverts the entire image stack—all its individual images—inverted. If you have an image on-screen and use this command, and then load another image, the second image is not inverted unless you use **Invert Displayed Image** on it. **Invert Displayed Image** does not invert any tracings.

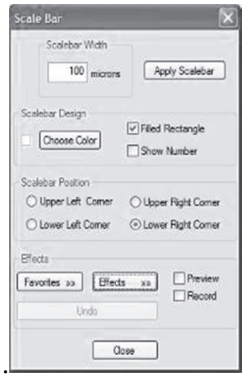
Invert Displayed Image does not make permanent changes to your image. If you save your image after using this command, your image is not saved as inverted.

Add Scalebar

Use this command to add a scalebar to the imaging area.

To add a scalebar

1. Click **Imaging>Add Scalebar.** Stereo Investigator displays the **Add Scalebar** dialog box



2. Type a value for the scalebar width, and click **Apply Scalebar.** Stereo Investigator displays the scalebar with your settings.

Scalebar options

You can change the following options:

Scalebar Design

Choose Color

Click Choose Color to display a color picker and select a color.

Fill Rectangle

Select to display the scalebar as a solid filled rectangle.

Show Number

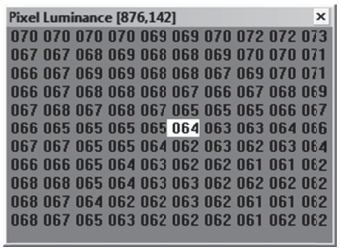
Select to include the scalebar width value.

Scalebar Position

Select a position for the scalebar.

Pixel Window

Displays the **Pixel Window,**



which only operates on an acquired (not live) image. The **Pixel Window** displays the luminance values of pixels in the vicinity of the crosshair cursor. Stereo Investigator displays the location of the pixel directly under the crosshair cursor in the Pixel Window titlebar.

For a grayscale image the luminance value is the same as the grayscale value, which is a number from 0 to 255 that describes the brightness of the image at any particular pixel. A black pixel has a luminance of 0. A

white pixel has a luminance of 255. Although this is a linear scale, the human eye does not work in a linear fashion. Subtle differences in low luminance values are not as easy for the eye to differentiate as subtle differences in high luminance values.

The luminance value of the pixel under the center of the crosshair cursor is highlighted in the middle of the window. The highlighted position is centered in the pixel window. The luminance values change as the crosshair cursor is moved. The size of the crosshair has no effect on the sampling of the image. As the cursor is moved to the edge of the image the values off the edge of the image are seen in the pixel window as dashes instead of numbers.

Averaging Luminance Values

Individual luminance values may not be as important as the average for a given area.

To obtain an average

1. Right click in the pixel window and select **Circular Crosshair Average.**
2. Use the mouse wheel or +/- keys to change the size of the circular cursor. Stereo Investigator highlights all values of pixels contained in the circular cursor area. The title bar of the pixel window shows how many pixels are included and the average luminance of those pixels, in addition to the location of the center pixel.
   The number of pixels is the number of green pixels plus 1 for the highlighted pixel in the center. Move the crosshair cursor to the edge of the image and see that the number of pixels drops as the green area is truncated by the edge of the image.

Grayscale images are composed of luminance values. Monochrome images are composed solely of pixels that are either 0 or 255. Paletted images and true color images are composed of pixels that have a triple of values. These values are a mix of red, green, and blue. These values are converted to luminance using the YIQ standard. Here, Y is the luminance: Y=(0.299)Red+(0.587)Green+(0.114)Blue The result is the same as if a black and white monitor was being used.

In addition to an average, the **Pixel Window** right-click menu lets you display the red, green, or blue channel of a true color or paletted image. The red, green, or blue pixel values are raw values—they are not multiplied by the values used in computing the luminance.

When do I use the Pixel Window?

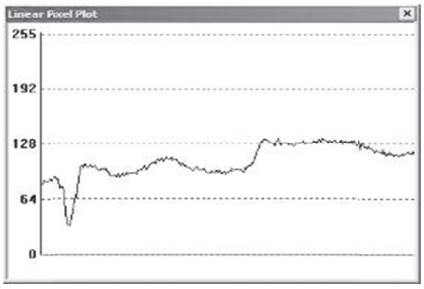
If you have image processing problems, the **Pixel Window** can be a useful tool. For example, when doing solid body tracing, Stereo Investigator looks for the edge of the solid body by searching out from the point where the cursor was clicked. It finds the edge by following a ray out from the starting point looking for sharp changes in the luminance values. Sometimes the solid body tracing creates odd shaped contours nowhere near the edge due to image noise. What appears at first to be an even dark soma may be full of salt and pepper noise. This is a fine sprinkling of pixels that are very bright or very dark. This type of noise is white noise. That means that the noise is random and not dependent on the values around the noise. The pixel window can be used to see if the image contains this type of noise.

Another problem in image processing is image segmentation. This means breaking the image into pieces, usually called the foreground and the background. The foreground is the part of the image that the researcher is interested in analyzing, the background is the remainder of the image. The Kodalith image effect is a standard method of doing image segmentation. It can be difficult to make it work if parts of the image are properly selected while other parts of the image are poorly selected. The pixel window can be

used to determine how the luminance values vary across the image. The idea is to learn why similar objects in different parts of the image do not have the same qualities. One possibility to investigate is if the illumination source needs to be adjusted to provide better illumination to all portions of the image.

Linear Pixel Plot

The **Linear Pixel Plot** window



displays a graph of the pixel luminance along a line segment, providing a graphical version of the information displayed in the pixel window. One of the ways to think of an image is as if the image represents a surface. The brighter areas are peaks and the darker areas are valleys. The line segment takes a slice though this 3-dimensional world and displays the results as a graph showing the"elevation" changes along the line segment.

Working with the Line Pixel Plot window

After opening the **Line Pixel Plot** window, a line appears over the selected image. If you don't see this line, the selected image may not be in the tracing window. Click the **Image Organizer** toolbar button and then click **Center Selected Image**. The selected image should now be visible in the tracing window and the line segment should appear over the image.

To move the line segment, move the cursor over the line segment until the cursor changes to a hand. Left click and drag the line segment. The orientation of the line segment and its length do not change. To move one end of the line segment, move the cursor over an end until a small white square appears. Left click and drag the end point to a new location. The length and orientation of the line are changed.

Move the line segment to a portion of the image considered to be background. Examine the line plot of this area. In general, background areas should look like horizontal jagged lines. The jagged appearance of the line is due to the noise component of the image. The line may not have an overall horizontal appearance. The greater the noise the less the line appears to be horizontal, and the harder it is to do image segmentation. Segmentation is the process of separating the things of interest, the foreground, from the rest of the image, the background.

Collect Luminance Information

This command only works on acquired images.

After choosing this command, all new contours that you draw record luminance information.

Stereo Investigator displays luminance information is displayed in the **Contour Measurements** window. To display contour measurements, click the **Contour Measurements** button.

To collect luminance information, you must have one or more closed contours, whether previously drawn contours or contours drawn while **Collect Luminance Information** is selected. However, once you deselect **Collect Luminance Information**, Stereo Investigator does not collect any information on new contours.

To record luminance information for previously drawn contours, click **Imaging>Collect Luminance Information**, then right click in the tracing window and select **Redo Luminance**. The status bar contains instructions to click inside a contour to collect luminance information for that contour. After clicking in a single contour, the **Redo Luminance** mode ends until you select it again.

Color Filters

The **Color Filters** command controls the display of color filters on acquired images, or changes color channels on confocal image stacks. The command also lists the shortcut keys that can be used to change color filters:

- None F9
- Red F10
- Green F11
- Blue F12

Use **Options>General Preferences>Imaging** tab to control whether the color filters are **Selected** or **Toggled** with the function keys. If toggling is chosen, multiple filters can be used simultaneously.

Max and Min Intensity Projection

The **Maximum** and **Minimum Intensity Projection** commands can be used to see an entire image stack in a single plane.

This technique collapses the intensity information from the 3D image volume into a single image plane. Either the maximum or minimum intensity value of each pixel along the z axis is projected onto the 2D plane. Maximum intensity projection should be used with light-on-dark images (such as fluorescence), while minimum intensity is designed for dark-on-light images (such as brightfield).

When you choose **Imaging>Maximum Intensity Projection** or **Imaging>Minimum Intensity Projection**, Stereo Investigator creates a new image and places it at the Z-depth of the top of the stack. To switch between the projection view and the stack, use the **Image Organizer**. Use **File>Image Save As** to save the projection image.

Delete Plane from Stack

Deletes the current plane from the image stack.

Deep Focus

As with the **Maximum and Minimum Intensity Projection** commands, you use **Deep Focus** to see an entire image stack in a single plane.

Deep Focus collapses the focus information from the 3D image onto a single image plane. That is, it takes the portions of the image that are in focus in each image of the stack and constructs a single image from that data.

When you select **Imaging>Deep Focus**, Stereo Investigator creates a new image and places it at the Z-depth of the top of the stack. To switch between the projection view and the stack, use the **Image Organizer**. Use **File>Image Save As** to save the projection image.

Image Montage

Starts the Image Montage module, an extension to the standard version of MBF Bioscience software, providing an additional capability of creating 2D and 3d image montages from images and image stacks.

For information and instructions, see the Image Montage module.

Automatic Object Detection

Stereo Investigator can automatically detect and outline and mark objects, reducing your workload.

While Automatic Object Detection can help with your work, you need to examine objects it outlines and marks for accuracy.

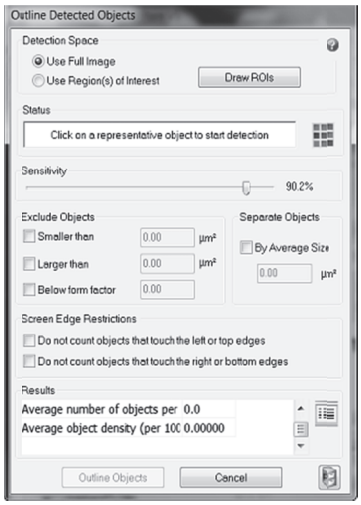
Outline Detected Objects

You must have an image loaded before you can use this command.

You can use this command to outline objects within the entire image or within Regions of Interest.

To outline detected objects with the entire image

1. Click **Imaging>Automatic Object Detection>Outline Detected Objects**. Stereo Investigator displays the **Outline Detected Objects** dialog box.



2. Select **Use Full Image**.
3. Select and adjust any of the parameters or continue to step 4. Note that each time you change a parameter, Stereo Investigator outlines detected objects. This is a preview display of what your

changes will produce. You must click **Outline Objects** to have Stereo Investigator use these parameters for results.

- **Sensitivity:** Increasing sensitivity restricts the detected objects closer to your representative sample color.
- **Exclude objects:** Excludes objects from detection that are **Smaller than** or **Larger than** the set micron value. You can also tell Stereo Investigator to exclude objects **Below form factor**,
- **Separate Objects By Average Size:** This setting can be used to make the count of detected objects more accurate. For example, if the average size is 10 μm^2 and Stereo Investigator finds an object approximately 30 μm^2, your detected object count will increase by 3 rather than 1.
- **Screen Edge Restrictions:** Tells Stereo Investigator to ignore objects that meet the touching criteria.

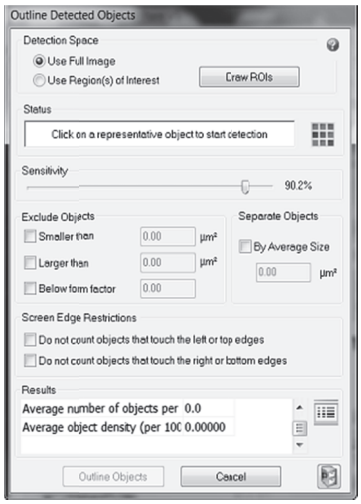
4. Click on a representative object to start. Stereo Investigator begins tracing detected objects.
5. **Outline Objects** commits the parameters you have adjusted to contours on screen. Make any adjustments to the parameters, and click **Outline Objects** again.

Can I change the color of the outline?: Click the color palette next to the Status line in the dialog box to display a color picker. You don't have to restart object detection to use a different color; just choose a new color and all detected objects use that new color.

6. Stereo Investigator shows you the results within the dialog box. For a list of contour measurements, click the **Contour Measurements** button, next to **Results**. Stereo Investigator displays the results in a new window.

To outline detected objects within Regions of Interest

1. Click **Imaging>Automatic Object Detection>Outline Detected Objects**. Stereo Investigator displays the **Outline Detected Objects** dialog box.



   1. Select **Use Region(s) of Interest**.
   2. Click **Draw ROIs**.

3. Trace a region or regions of interest on the image. Stereo Investigator will only detect objects within these regions.
4. When you have traced your region or regions, click **Finished ROIs**.
5. Select and adjust any of the parameters or continue to step 6. Note that each time you change a parameter, Stereo Investigator outlines detected objects. This is a preview display of what your changes will produce. You must click **Outline Objects** to have Stereo Investigator use these parameters for results.
    - **Sensitivity:** Increasing sensitivity restricts the detected objects closer to your representative sample color.
    - **Exclude objects:** Excludes objects from detection that are **Smaller than** or **Larger than** the set micron value. You can also tell Stereo Investigator to exclude objects **Below form factor**,
    - **Separate Objects By Average Size:** This setting can be used to make the count of detected objects more accurate. For example, if the average size is 10 μm^2 and Stereo Investigator finds an objrct approximately 30 μm^2, your detected object count will increase by 3 rather than 1.
    - **Screen Edge Restrictions:** Tells Stereo Investigator to ignore objects that meet the touching criteria.
6. Click on a representative object to start. Stereo Investigator begins tracing detected objects.
7. Make any adjustments to the parameters, and click on an object to start detection. The object does not have to be within a region of interest. Stereo Investigator detects and outlines the objects.
8. Stereo Investigator shows you the results within the dialog box. For a list of contour measurements, click the **Contour Measurements** button, next to **Results**. Stereo Investigator displays the results in a new window.

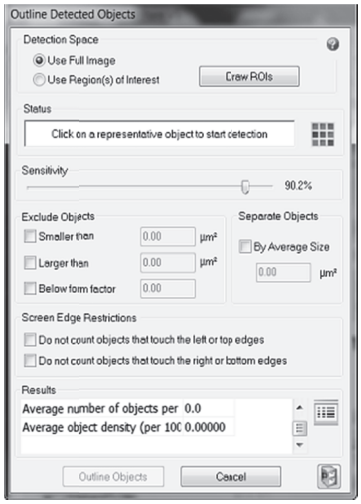
Mark Detected Objects

You must have an image loaded before you can use this command.

You can have Stereo Investigator mark detected objects automatically, within the entire image or within Regions of Interest.

To mark objects with the entire image

1. Select a marker from the Marker bar.
2. Click **Imaging>Automatic Object Detection>Mark Detected Objects**. Stereo Investigator displays the **Mark Detected Objects** dialog box



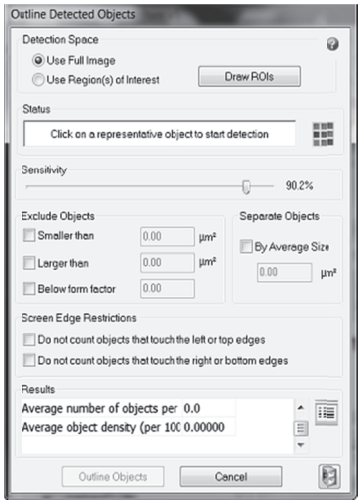
3. Select **Use Full Image**.
4. Select and adjust any of the parameters or continue to step 5. Note that each time you change a parameter, Stereo Investigator marks objects. This is a preview display of what your changes will produce. You must click **Outline Objects** to have Stereo Investigator use these parameters for results.
    - **Sensitivity:** Increasing sensitivity restricts the marker placement to objects closer to your representative sample color.
    - **Exclude objects:** Excludes objects marking that are **Smaller than** or **Larger than** the set micron value. You can also tell Stereo Investigator to exclude objects **Below form factor**,
    - **Separate Objects By Average Size:** This setting can be used to make the count of detected objects more accurate. For example, if the average size is 10 μm^2 and Stereo Investigator finds an object approximately 30 μm^2, your detected object count will increase by 3 rather than 1.
    - **Screen Edge Restrictions:** Tells Stereo Investigator to ignore objects that meet the touching criteria.
5. Click on a representative object to start. Stereo Investigator begins marking objects.
6. Make any adjustments to the parameters, and click **Mark Objects** again.

Can I change the color of the outline?: Click the color palette next to the Status line in the dialog box to display a color picker. You don't have to restart object marking to use a different color; just choose a new color and all marked objects use that new color.

7. Stereo Investigator shows you the results within the dialog box. For a list of contour measurements, click the **Contour Measurements** button, next to **Results**. Stereo Investigator displays the results in a new window.

To mark objects within Regions of Interest

1. Click **Imaging>Automatic Object Detection>Outline Detected Objects**. Stereo Investigator displays the **Outline Detected Objects** dialog box



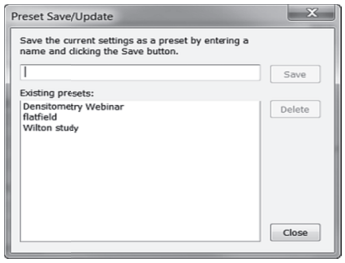
1. Select **Use Region(s) of Interest**.
2. Click **Draw ROIs**.
3. Trace a region or regions of interest on the image. Stereo Investigator will only place markers within these regions.
4. When you have traced your region or regions, click **Finished ROIs**.
5. Select and adjust any of the parameters or continue to step 6. Note that each time you change a parameter, Stereo Investigator marks objects. This is a preview display of what your changes will produce. You must click **Outline Objects** to have Stereo Investigator use these parameters for results.
    - **Sensitivity:** Increasing sensitivity restricts the marker placement to objects closer to your representative sample color.
    - **Exclude objects:** Excludes objects marking that are **Smaller than** or **Larger than** the set micron value. You can also tell Stereo Investigator to exclude objects **Below form factor**,
    - **Separate Objects By Average Size:** This setting can be used to make the count of detected objects more accurate. For example, if the average size is 10 μm^2 and Stereo Investigator finds an object approximately 30 μm^2, your detected object count will increase by 3 rather than 1.
    - **Screen Edge Restrictions:** Tells Stereo Investigator to ignore objects that meet the touching criteria.
6. Click on a representative object to start. Stereo Investigator begins marking objects.
7. Make any adjustments to the parameters, and click on an object to start placing markers. The object does not have to be within a region of interest. Stereo Investigator places markers on the objects.
8. Stereo Investigator shows you the results within the dialog box. For a list of contour measurements, click the **Contour Measurements** button, next to **Results**. Stereo Investigator displays the results in a new window.

Using Presets

You can use presets to apply the same parameters to many images. You can save presets , edit them, and load previously saved presets.

To save or delete presets

1. Click the Presets button and choose **Edit** Presets. Densita displays the Preset **Save/Update** dialog box.



2. Type a name for the preset, and then click **Save.**
   -or-Select an existing preset and click **Delete** to remove it.

To load an existing preset

- Click the Presets button and choose a preset from the list.

Solid Body Tracing

Solid Body Tracing automatically traces a contour that follows the outline of a solid object such as a cell body. The entire object must be visible in the current field-of-view, so select an appropriate objective before beginning. This technique depends on a contrast difference between the cell body and the background, and upon a body edge that is in focus. Focus carefully before acquiring the image.

Solid Body Tracing works only on acquired images, and it works best on monochrome images. Use the Kodalith feature of Image Processing for Kodalithing.

Using Kodalith on an image

Kodalithing changes an image to black and white. This is not the same as grayscale, which consists of gradations of black and white. In a Kodalithed image, every pixel is either black or white. The program then uses this binary information to do analysis of particle and contour locations.

Currently, this procedure only works with monochrome images, so you need to convert the image before you start solid body tracing.

To convert to monochrome

1. Click the **Image Processing** button
   -or-
   click **Imaging>Image Processing.**

2. In the **Image Processing** dialog box, click **Effects>Color Transform>Kodalith.**
   A preview window appears in the upper left corner of the image. Drag the window and adjust the size until it covers a portion of your object.
3. Use the slider bar in the **Image Processing** dialog box to adjust the Kodalithed image until the object you want to trace appears black against a white background.
4. Click **Apply Kodalith** to see how the effect looks on the whole image.
5. If it is satisfactory, click **Close.** If it is unsatisfactory, click **Undo Kodalith**, and readjust the parameters until the results are satisfactory.

How to perform Solid Body Tracing on an image

Be sure that a reference point has been placed, and that the Contour Mapping mode is active, or cell body is chosen if you are in Neuron Tracing Mode.

1. Click **Imaging>Solid Body Tracing**, or click the **Solid Body Tracing** button. You are now in **Solid Body Tracing** mode, and remain in this mode until it is deselected by clicking the button again, or clicking **Imaging>Solid Body Tracing**.
2. Right click on the image to open the **Solid Body Parameters** dialog box. If you want to use a previously saved preset, click the **Presets** button and select a preset from the list.
3. Left click within the object to be automatically traced.
4. In the **Solid Body Tracing** dialog box, click **Trace**. Stereo Investigator draws a test contour around the selected object..
5. Adjust the parameters until the contour correctly traces the boundary of the selected object.

   You can use the Editing Mode while in Solid Body Tracing Mode to delete undesired contours. When you enter the Editing Mode, the dialog box disappears, but reappears when the Editing Mode is closed, and you are still in Solid Body Tracing mode.

6. Click on a few other objects to make sure these parameters are generally effective.
7. Click **Close** when the appropriate parameters have been determined.
8. Click on each of the objects in the field-of-view to automatically trace them. around each one.
9. In the **Solid Body Tracing** dialog box, click **Trace**. Stereo Investigator draws a contour around each one.

   You can select the Contour Measurements window to view the cross-sectional areas and perimeters of these objects.

Solid Body Tracing Parameters

Automated tracing may not appear initially successful. Adjusting the Solid Body Tracing parameters can increase accuracy.

The Tracing Algorithm: How Does It Work?

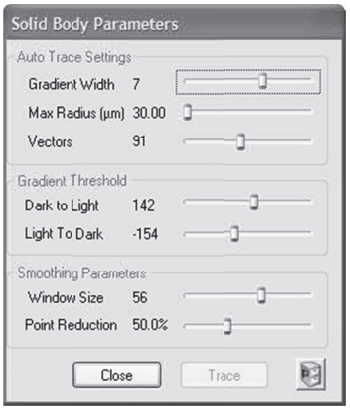
Correct adjustment of these parameters is necessary to help the tracing algorithm correctly identify edges of the selected object. It can be helpful at this point to have a general idea of how this algorithm works: When you click on an object, a number of radial vectors are drawn from the selected point out to the maximum radius. A small sliding window of contiguous pixels along each radial vector is examined for a

change in luminance. The point at which the most significant change is encountered is considered the edge of the object along that particular vector. When each vector has determined all edge points, these edge points are connected to make the outline contour. As you can see, placement of the central point is important to how the algorithm "sees" the contour. Be sure the central point is in line with any areas of the object that protrude from the central area if you want these protrusions traced effectively.

If you click on a different center, the radial vectors emanate from this new point, explaining why results may vary widely in different runs of this protocol.

To adjust Solid Body tracing parameters

- Right click within the tracing window to display the **Solid Body Tracing** dialog box.



Auto Trace Settings

Gradient Width

This setting determines the size of the sliding window of pixels that the algorithm uses to detect variations in luminance between the object and background. A good starting value is 5. If the edge of the object is particularly fuzzy, you may need to use a value greater than 5. The number should be less than the number of pixels that separate two distinct objects.

If the object contains spots of background color within its interior, the gradient width should be larger than the number of pixels needed to span these spots, otherwise the spots are interpreted as boundaries.

Max Radius

Determines the maximum distance from the clicked starting point that the radial vectors extend. This determines the maximum size of objects traced automatically.

Vectors

Determines the number of vectors used to search for edges. 70 vectors is usually sufficient for small reasonably round objects. As the value is raised, irregularities in the shape of the boundary are followed more closely, but the procedure may take more time.

Gradient Threshold

This value specifies the pixel luminance gradient at which a boundary is considered to occur. **These values are important if you are not using a monochrome image.**

Dark to Light

A value of 20 indicates that the average luminance values outside of an object should be at least 20 units higher than those inside when tracing a dark object against a light background.

Light to Dark

A value of -20 indicates that the average luminance values outside of an object should be at least 20 units lower than inside when tracing a light object against a dark background.

Smoothing Parameters

Smoothing minimizes jagged irregularities in a contour. This can be necessary due to minor errors in the automated tracing.

Window Size

This parameter modifies the placement of any outlier boundary points. If this value is greater than one, extreme outliers (isolated points that are far from the mean) are brought toward the mean boundary distance from the selected point in the interior of the object. Leave this value at one if you have a highly irregular contour, and want to capture the actual shape most accurately. Increase the value for objects with more even outlines.

Point Reduction

Specifies the percent reduction in the number of points in a contour. If this parameter is set to 50%, then every pair of sequential points along the contour outline are replaced by a single point which is an average of the two. Keep this set to zero if you want to accurately trace an object with a convoluted outline. Point reduction also acts to smooth traced outlines.

Working with presets

You can save presets , edit them, and load previously saved presets.

To save or delete presets

1. Click the **Presets** button and choose **Edit Presets**. Stereo Investigator displays the **Preset Save/Update** dialog box.
2. Type a name for the preset, and then click **Save.**
   -or-Select an existing preset and click **Delete** to remove it.

To load an existing preset

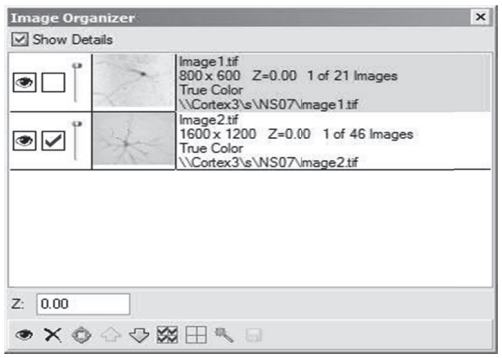
- Click the **Presets** button and choose a preset from the list.

Image Organizer

The **Image Organizer** shows the location and status of each of the images that are open in the current tracing window, and contains several features for manipulating the appearance of the images.

The Image Organizer Interface

The images are listed in the order in which they were opened. The most recent image opened is listed first, and appears in front of the other images where they overlap. Each layer can contain only one image, so the terms layer and image can be used interchangeably.



Column 1

- The eye icon indicates the visibility of the layer. Click the eye icon to turn visibility for this image on or off.
- When a check mark is present, the image is grouped with other checked images, for commands such as **Move Image, Move Image and Tracing, Image Processing,** etc. Click to turn grouping on or off.

An image must be checked in order to move the image with either **Move Image** or **Move Image and Tracing** If your image is not moving when a joystick function is used, be sure that the image is checked in the **Image Organizer**. Only the checked images are affected by the move operations.

- Use the **Transparency Slider** to adjust the transparency of an image. This tool is extremely valuable in aligning images imported separately.

The **Transparency Slider** does not affect markers or tracings. Use the **Hide Tracing** button if you want to hide markers or tracings.

Column 2

- A thumbnail view of the image. This image changes if you apply any image effects to it.

Column 3

- This column lists the image file name. If **Show Details** is checked, you will also see the resolution, your location within the stack (if the image is part of the image stack), what type of image (True Color, Grayscale, etc.), and the location of the image.

The **Show Details** checkbox toggles display of the information associated with each image.

Z is a Z-depth adjustment tool. Type a value in the text box for the Z-value.

Click and drag up or down anywhere in the row to move the layers in respect to one another. For example, clicking on the bottom row, holding down the mouse, and dragging it to the top makes that layer the new "top" layer.

The toolbar at the bottom of the **Image Organizer** window contains the following commands.

	Toggles visibility for checked images.
	Discards checked images.
	Centers image in the Tracing window.
	Moves image up or down in Image Organizer list.
	Checks all images.
	Unchecks all images
	Applies image processing effects to the checked images.. This only works for images of the same type (true color, paletted, grayscale, or monochrome), and the preview is only seen in the selected image. When you click this button, the image effect preview is only seen on the selected layer. The selected image is also the image that is used in the **File>Image Save, File>Image Save As, Pixel Window, Histogram** window, and the **Line Plot** window. The selected image is not the same as the checked images. More than one image can be checked (for deletion, application of image effects, and hiding of images), while only one image can be selected at a time.
	Saves changed images.

Options Menu

Stage Setup

Use this command to specify the communications and operating parameters of the stage controller. This dialog box has one or more tabbed pages, depending on which stage is chosen on the **Stage Type** tab.

WARNING: Choosing the wrong stage or the wrong settings can damage to the stage, microscope, or other equipment!

If you think you need to make any changes to your stage setup, please contact MBF Bioscience Product Support for instructions.

Video Setup

This command sets the type of video system used to display a live video feed of the microscope's field of view in the application window. Stereo Investigator displays the live image in a non-destructive overlay; this means that each pixel of a particular color, referred to as the key color, is replaced by live video. This lets you trace, count, and normally operate your computer while the live video updates in Stereo Investigator.

More about Video Setup

Stereo Investigator supports the following:

- A video camera connected to the computer via a video frame grabber card.
- A digital camera connected via IEEE 1394 Firewire or a digital frame grabber card.

The video camera/frame grabber setup generally displays live video at a fast rate but lower resolution. However, digital cameras typically update live video at slower rates, but have substantially higher resolutions.

Which cards and cameras are supported?

Stereo Investigator supports a vide number of cards and cameras. We are always evaluating new hardware and adding support. If you are using a card or camera not listed here, please contact MBF Bioscience Product Support for information and assistance.

For a list of the latest supported cards and cameras, please see the Online Help for this topic.

Microscope Setup

You use this command to set up microscopes.

WARNING: Choosing the wrong microscope or the wrong settings can cause damage to the stage, microscope, or other equipment.

If you think you need to make any changes to your microscope setup, please contact **MBF Bioscience Product Support**.

We currently support the following microscopes. Note that we are continually evaluating new hardware, and may add to this list. Please contact MBF Bioscience Product Support if you have any questions.

- Olympus IX2/BX2
- Zeiss MTB Supported Scopes
- FLUOVIEW
- Nikon
- Leica AHM Supported Scopes
- SAR Procyon
- CARV2
- Prior Controller Without Stage

- MBF PCI Controller Without Stage
- Sutter Instrument Co. Controllers (Lambda SC)
- Shutter Control
- OptiGrid

External Image Source Setup

Use to set up the use of an external image source. See this command int the software for help and information.

Message Device Setup

Use this command to define a message that can be used in a **Device Command Sequence.**

To add a message:

1. Click **Options>Message Device Setup**.
2. In the **Message Device Setup dialog** box, click **Add.** Stereo Investigator displays the **Message of Message Device** dialog box.
3. Type a name for your message and then type the message text you want displayed to the user.
4. Click **OK**. The message is saved.

To edit a message:

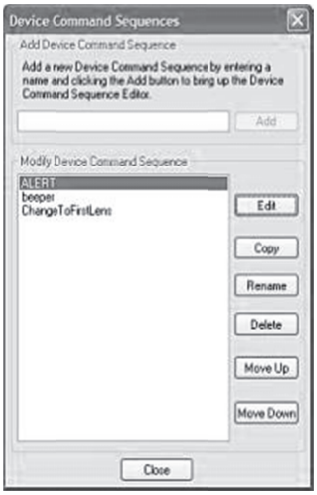
1. Click **Options>Message Device Setup**.
2. In the **Message Device Setup** dialog box, select a message and then click Edit. Stereo Investigator displays the **Message of Message Device** dialog box with your message and text.
3. Make any changes to the text, and then click **OK**. Your changes are saved.

Device Command Sequence Setup

You can define a Device Command Sequences so that you can select and issue commands to a device or devices, and all the mechanical pieces of the microscope (for example the filter cube, the shutter, the filter wheel, the disc, etc.) are placed in the right position for viewing or imaging. .

To create a Device Command Sequence

1. Click **Options>Device Command Sequence Setup,** Stereo Investigator displays the **Device Command Sequence** dialog box.



2. In the **Add Device Command Sequence** text box, type the name of the sequence to be defined. For example, *TRITC*.
3. Click **Add.** Stereo Investigator displays the **Device Command Sequence Editor** dialog box.

4. From the **Device** pull down menu, choose the device to be added to the Device Command Sequence
5. From the **State** pull down menu, choose the state to be added to the Device Command Sequence.

Note: The Test State button lets you test if the particular device is communicating with the computer.

6. Once the **Device** and **State** are selected click **Add to Sequence** to add the sequence to the list.
7. Repeat for all the devices necessary to complete the device command sequence.

Working with Device Command Sequences:

- To delete an individual device, select the device from the list and click **Delete**.
- To reenter the device, select the device from the **Device** pull down menu, select the correct state from the **State** pull down menu and click **Add to Sequence**.
- The order of the individual devices can be rearranged by selecting on the individual device and selecting either the Move Up or Move Down button.

General Preferences

Use this command to set or change preferences for using the Stereo Investigator software.

Cursor

You can change the color, thickness, and size of the cursor. In addition, the bottom of this page shows the keyboard and which keys adjust the size of the crosshair and circular cursors.

Crosshair Color

- **Set Color:** Click to select a new color for the crosshair cursor. Note, however, that the cursor may not actually be drawn in this color when a live video image is displayed. If the cursor does not stand out well when viewing a live video image, experiment with the cursor color until it is clearly visible. Try picking various colors—picking a new cursor color changes the color of the cursor, but may not change it to the selected color. Experiment with the colors until a color selection makes the cursor stand out clearly against the live video image.

Circular Cursor Size

- **Diameter:** Sets the circular cursor size, which you in microns. The circle is drawn as close as possible to the specified size, with the micron to pixel ratio of the current lens being the limiting factor in its accuracy. Stereo Investigator always records the circular cursor size during tracing. In order to view the line thickness, you must enable it with **Options>Display Preferences>View>Thickness**.

  Use the + and – keys on the numeric keypad to increase and decrease its size. You can also use the mouse wheel to adjust the size. If the mouse wheel is being used to focus the microscope, then hold the CTRL key down to change the size of the circular cursor.

Crosshair Size

It is often useful to set the cursor to a known size; this makes rapid comparison of objects on the screen possible. You can set the crosshair size in pixels or microns. If you choose microns, the cursor changes size when a new lens is selected. If you choose pixels, the crosshair remains a constant size, even when changing lenses. You can also use the arrow keys to adjust the crosshair size. Note that the left and right arrows control the thickness of the cursor lines. Make sure NUMLOCK is turned off if the arrow keys on the numeric keypad are used.

Blinking

When tracing, a special marker, called the current position marker, is shown blinking at the last point traced. You set the shape, size, and speed at which the cursor revolves or blinks in this tab. These options provide a balance between not obscuring the image, locating the current position, and clarity. For example, a slow blinking current position marker may be hard to locate, but it spends very little time blocking structures on the screen. A wheel shaped current position marker can blink more quickly, but the exact position of the previously traced point may not be as clear.

Style

- None: No current position marker is shown.
- **Circle:**Display the current position marker as a circle.

- **Cross Hair:** Display the current position marker as a crosshair.
- **Wheel:** Display the current position marker as two revolving spokes of a wheel.
- **Cursor:** Display the current position marker as a tangent line revolving around the perimeter of a circle whose size is determined in the Radius edit box.

Radius

The radius of the current position marker in pixels, independent of the lens.

Speed

The blinking rate is adjusted by moving the slider from slow to fast. A slow blink means that the current position marker is seen for a long time and then not seen for an equally long time. Speeding up the blink shortens both the visible time and the time the marker is off.

Movement

AutoMove Settings

These options are used in stage movement If a point is placed outside the AutoMove Area, the stage moves to center the point in either the tracing window or in the AutoMove Area.

- **AutoMove On:** This determines whether or not the **AutoMove** area is activated. The same setting can be changed with **Move>AutoMove**. The **AutoMove** button also toggles activation of the **AutoMove** area.
- **Center Cursor in AutoMove Area:** This determines if the program centers the cursor in the **AutoMove** area after an **AutoMove** operation. If this box is not checked, the cursor position is not changed when an **AutoMove** occurs. Most users prefer to leave this option turned off.
- **Center Data in AutoMove Area:** The center of the tracing window is normally used as the center point when repositioning with the **AutoMove** area. Select this option to use the center of the **AutoMove** area instead of the center of the tracing window when repositioning the stage. These can be different since the **AutoMove** area is not necessarily centered in the main tracing window. If the video image does not take up the entire tracing window, the **AutoMove** area is centered over the image. A video image is placed in the upper left corner of the tracing window. Depending on the size of the video image, centering data in the **AutoMove** area may be a better method of handling automatic stage movements.

Field Movement Size

This set of options determines how the stage is moved when using either the arrow buttons on the Main toolbar, the **Move>Field** menu options, or **Move>Meander Scan**.

- **% of Screen Size:** The amount of movement can be set to a fixed percentage of the size of the tracing window. The default at the time of installation is 75 percent of the size of the tracing window. This size is convenient and balances overlap with efficiency in covering an area. The percentage can be set from 10 to 100 percent. To exceed these bounds use the user specified option.
- **AutoMove Area Size:** Setting the Field Movement Size to the AutoMove Area Size allows you a visual means to set the Field Movement Size. Movements in X are the same as the width of the box

and movements in Y are the same as the height of the box. With this option selected, click **Options>Define AutoMove Area** to set the **Field Movement Size**.

- **User Specified:** The most general option is the user specified field movement sizes. They are entered in microns. Unlike the percent of screen size and the **AutoMove** area options, the user specified values might not make sense for all lenses. The previous options move different distances depending on the lens in use. The user specified step sizes are fixed micron values that are the same for all lenses.

Mouse Wheel

If Focus with mouse wheel is not checked, the mouse wheel changes the size of the circular cursor. If Focus with mouse wheel is checked, each click of the mouse wheel moves the Z-axis the distance specified in **the** Z distance per wheel click field.

The function of the mouse wheel can be toggled by holding down the CTRL key while rotating the mouse wheel. If Focus with Mouse Wheel is checked, then holding down CTRL while rotating the mouse wheel causes the circular cursor to change in size.

AutoSave

- Select **Enable Auto Save** to write automatic backups of tracing data.

Triggers

Automatic backups happen whenever certain events (called triggers) occur.

- Time Interval: Indicates the number of minutes between periodic backups. A time interval of 0 means that timed intervals are not used.
- Data Points: Determines how many points can be entered in the data file before a backup is automatically initiated. Set the value to 0 to disable this option. Counting data points is really counting data point events. Left clicking the mouse enters a point. Each click counts as a single data point event. The continuous tracing method enters many points in a single sweep. Each sweep counts as a single data point event. The number that is entered is related to steps in the work and not the number of individual pieces of data that are created. Enter the value accordingly.

Lens

Use this tab to set options to compensate for differences between objective lenses.

Corrections

If Parcentric/Parfocal calibration has been performed, these options can be enabled so that corrections are automatically applied whenever you change lenses. The Parcentric and Parfocal correction procedure is described in the section Lens Calibration.

- **Enable Parcentric:**When enabled, Stereo Investigator automatically compensates for collimation differences between lenses.
- **Enable Parfocal:** When enabled, Stereo Investigator automatically compensates for differences in focal planes between lenses.
- **Center View:** Centers the lens view.

Numerical Formatting

Stereo Investigator collects all information in microns. Options include the choice of measurement units and numerical precision.

The display of measurement units can be chosen in microns, millimeters, or centimeters, with precision values ranging from 1 to 6 significant digits.

Selecting the use scientific notation option formats the display of all numeric values with the number of digits specified by precision followed by the appropriate exponent.

A number that is stored internally as 109814.37 is displayed as 1.09814e5 if precision is set to 6. If precision is set to 3 it is displayed as 1.10e5. Numbers are rounded to the number of digits specified.

Length Measurements

Length measurements can be displayed in microns, millimeters, or centimeters.

Area Measurements

Area measurements can be displayed in square microns, square millimeters, or square centimeters.

Volume Measurements

Volume measurements can be displayed in cubic microns, cubic millimeters, or cubic centimeters.

Angle Measurements

Angle measurements can be displayed in degrees or radians.

Miscellaneous

Recent Files

The number of recent files shown in the recent files lists at the bottom of the File menu is specified here. The default is 4. If there are more than 4 files that you frequently use, you can set the value to that number of files, and then open each file with a single click from the File menu.

Use Pen Buttons

This option enables the use of the pen buttons on the stylus of a Wacom data tablet. When selected, the front and back of the stylus button are used to change the circular cursor size, and the right click is then assigned to the button on the end of the pen (the 'eraser'). The Wacom defaults are for the front of the button (called a 'switch' by Wacom) to be assigned to right click and the back assigned to left double click.

Wacom Tablet Stylus Settings

To use this option, the settings on the Wacom stylus must be changed in the **Wacom Tablet Properties** dialog box, shown here. On the Tool Buttons tab of the Wacom, set Eraser Function to Erase, and set both Switch Functions to Ignored.

Suppress warning for saving single image of a stack:

If **File>Image Save As** is used to save a single image of an image stack, a warning appears informing you that you are only saving a single image of the stack, and that you should use File>Image Stack Save As if what you meant to do was to save the entire stack. If you are saving multiple single images, you may want to suppress this warning.

Enable use of a timer device

Enables use of a timer device for some operations.

Luminance

The **Video>Collect Luminance** command is used to collect luminance information. Select the details of the collection process here. The more information that is collected the larger the tracing files. Five pieces of information are always collected: The mean luminance, the standard deviation, the minimum luminance, the maximum luminance, and the total number of pixels. The histogram of the collected luminance values can also be stored. The histogram provides an excellent representation of the results without markedly increasing the size of the tracing files. The sampled image can also be saved. The image provides all of the information, but at the cost of much larger tracing files.

Save Image Histogram

The histogram of the luminance values can be saved along with the basic five pieces of luminance information. If the original image was a color image the color pixels are transformed into luminance values. A histogram of these luminance values describes the distribution of luminance values in the sampled area. The increased information has a minor impact on the size of the tracing files.

The luminance histogram is calculated as (.299 * Red) + (.587 * Green) + (.114 * Blue) unless separate color channels are selected.

Save Image

Saving the image provides the most complete information about the area sampled with the **Collect Luminance** command. This completeness can greatly increase the size of the tracing files if the contour is large. Choosing **Save Image** does not save the histogram, but gives future access to the histogram since the image itself if saved and the histogram can be generated again.

Saved Image Format

The images can be saved in two different formats. The **Save As Luminance Image** option converts the original image to luminance values before storing the image. The other option is the **Save As Original Image. Save as a Luminance Image** uses 1/3 less memory than **Save as Original Image** when color images are used.

The luminance options are intended to provide a broad range of options that balance information content with storage overhead.

Imaging

These options control the display and loading of imported images.

- **Marquee Around Current Image:** A marquee is drawn around the current image when this option is selected. The current bitmap is the only image that is used in many image processing operations. For example, the histogram window, pixel window, and other operations are all based on the current image. The other way to identify the current image is to inspect the **Image Organizer**, which shows the current image thumbnail with a black background for the adjacent text).
- **Load all stacks at same Z:** When enabled, all image stacks are loaded with the first image of the stack at the same Z-axis position. Image stacks are usually loaded with the first image at the current Z position, with the images in the stack placed at regular intervals in Z from the first image. If this

option is not selected, subsequent images are loaded at the current Z position. For example, if an image has been loaded and you have paged down to the 4th image in the stack, a new stack is loaded so that the top of the new stack is at the level of the 4th image of the first stack. Selecting this option causes all subsequent image stacks to be aligned with the top of the first image stack.

- **Show Images in Where Is mode:** When enabled, the acquired and imported image thumbnails are shown in their appropriate locations when the **Where Is** mode is being viewed.
- **Turn Off Preview on Apply:** This option affects the viewing of **Image Processing** effects. If this option is checked, the preview area will disappear after an **Image Processing** effect has been applied. It can be turned back on by checking the **Preview** box in the **Image Processing** dialog box.
- **Turn On Preview On New Effect:** This option affects the viewing of **Image Processing.** If this option is checked, a preview window automatically appears whenever a new **image Processing** effect is selected.
- **Select Color Filters with F9-F12 and Toggle Color Filters with F9-F12:** Color filters can be either selected or toggled with the function keys F9-F12. If using the function keys to select a filter, only the color filter selected is used when that function key is pressed. If function keys are used to toggle filters, multiple filters can be used at the same time in variable combinations. Function keys are assigned as follows: F9=none, F10=red, F11=green, F12=blue.

Image Averaging

Image Averaging replaces each image acquire with n acquires and average these n acquires into a single image. Use this option if your camera generates a lot of noise in the image. Enter the number of images to be averaged, starting with a small number and gradually increasing until the noise reduction is satisfactory.

Image File Reading and Writing Protocol

Use these options to control whether configuration files for image display are written as separate external files, and whether to prevent the configuration from being written into the image file if the image hasn't changed.

The first option tells Stereo Investigator to write any MBF-specific information to a separate XMP file, and to use this file when reading an image into Stereo Investigator, if the file is available. MBF-specific information includes data about the Data DIB, Display DIB, and other information about the image file and how Stereo Investigator treats it.

The second option directs Stereo Investigator that when saving only image display adjustments, write these changes to the external XMP file, and not the image file.

Extended image data maintained in memory

You can direct Stereo Investigator on how to deal with extended image data. Choose **All, All acquired, All unsaved,** or **None.** In most situations, **All unsaved** is a good choice. Remember than every time you change an image, the extended data about the image also may change. If you are having memory issues, you can adjust this option.

When Image Bit Depth is Unknown

If the image bit depth can't be determined, you can tell Stereo Investigator what to do. You can tell it to use Max Pixels, set and use a fixed bit depth, or always ask you what to use.

Other Options

- **Save Images when they are acquired:** Select this option to automatically save the acquired images to a defined path. You can also give each acquired image a base name, for example, *luciferyellowOLG*. As each image is saved, Stereo Investigator uses this name and a number, counting up.
- **Remove Off-Screen Image Stacks From Memory:** This feature can decrease the memory demands that large image stacks can put on the system by only using memory for images that are currently being displayed. If this option is enabled, the images that make up an image stack will be unloaded from memory under the following conditions:
    - The image stack has not been modified since being loaded
    - The image stack is not visible in the current field of view
    - The image stack is not selected in the image organizer

The image stack will be reloaded when an image stack is performed on the stack, when the image stack is selected in the **Image Organizer**, or when the image stack is moved so that it is in the current field of view.

- **False Color Single Channel Images:** Select when working with single-channel images where you want Stereo Investigator to use false coloring.
- **Remove Off-Screen Image Stacks From Memory:** Checking this option will make some image actions faster, but it may take longer to load any off-screen image stacks.
- **Use Virtual Image mode when possible:** Check to use this mode where possible.

Acquire Setup button: Displays the **Acquire Setup** dialog box.

Zooming

Aspect ratio after zoom operation

Select one of the options to tell Stereo Investigator which aspect ratio—the current image or the current lens—to use.

Click **Notify user when operation changes aspect ratio by more than 10%** if you want to notify the user of this condition.

Tracing

The various tracing modes and their tolerances can be changed here.

You must select a reference point before this tab is available.

Tracing Mode

There are three tracing modes to trace contours and neuronal processes. The tracing mode can be changed during active tracing by selecting a new mode from the right click menu.

- **Simple Click Tracing:** This method of tracing involves positioning the cursor along the object to trace and clicking the left mouse button, then moving the cursor to a new position and clicking the left mouse button again to draw a line segment between the two points. This is done repeatedly until the entire contour has been traced.

- **Rubber Line Tracing:** This tracing method is similar to Simple Click Tracing, except a rubber band line is drawn from the last point traced to the cursor. This provides a visual indicator of where the next line segment will be drawn.
- **Continuous Tracing:** In this tracing mode line segments are automatically drawn as you move the cursor while holding down the left mouse button.

As you trace, the stage and tracing only move if you click and release the left mouse button outside of the **AutoMove** area. In the continuous tracing mode, the tracing can pass outside of the **AutoMove** area without moving as long as the left mouse button is held down.

Continuous Tracing Tolerance

This adjustment controls how smooth the tracing is when using **Continuous Tracing** mode. Selecting **Fine** results in the smoothest curves, but places more points in the data set. Selecting **Course** results in fewer data points for a given curve, but the curve is not as smooth.

The smoothness of the traced curve must be balanced against the overhead of more data points. Curves with many points are harder to edit.

Z Deviation Warning

The Z deviation warning is intended to detect problems in tracing dendritic structures. Tracing branched structures involves many steps during which mistakes can introduce large jumps in Z. Since Z is the dimension going into and out of the screen it is not always clear that points are not close to each other in Z. The warning discriminant is a micron value. A warning is flagged if the difference of the Z coordinates of two sequential points exceeds this discriminant.

Double Click On Mouse

Select to close a contour with a double-click.

Stereology Preferences

In addition to General Preferences and Display Preferences, Stereo Investigator also has a separate set of Stereology Preferences.

SRS

Controls Systematic Random Sampling options.

Region of Interest Options

- **Exclude Nested Contours:** Don't include nested contours when working with a region of interest.

Sampling Reminders

Displays a series of reminders displayed by Stereo Investigator when working with various functions.

Z Meter Usage

- **Use Relative Mode When Counting And Measuring Section Thickness:** Self explanatory.

Counting Frame

The counting frame is the graphical overlay used in a probe run to show the outlines of the area to be sampled. In general, a counting frame has two red edges and two green edges. The red edges are lines of exclusion, extending to infinity. Any cell or object that touches the red line, crosses the red line, or is outside of the counting frame is not counted. Any object inside the counting frame, touching a green line from either inside or outside the counting frame, or crossing the green line is counted.

If an object touches or crosses both a green line and a red line, it is not counted.

Several profiles are drawn to indicate which ones are and are not counted. A red X in the profile indicates that it is not counted, a green check mark indicates that it is counted.

Options

Many of these options are self explanatory.

Definition Options

- **Force Counting Frame To Be Square:** Forces the counting frame to a square shape.

Display Options

- **Display Counting Frame With Extended Exclusion Lines:** Extends the exclusion lines beyond their normal range.
- **Clip Combined Probes To Counting Frame:**
- **Mark Center Point of Counting Frame:** Adds a center point to each counting frame.
- **Hide Counting Frame When Using Local Probes:** Does not display the counting frame whenever a local probe is in use.
- **Use Not In Z Limit Color When Above or Below Disector:** When you are above or below the disector, Stereo Investigatorwill use a special color to alert you to this.

Counting Frame Colors

Click a color button to change the color.

- **Show Section Cut Thickness:** Displays section cut thickness.

Other Options

- **Restrict Placing Markers To Inside of Counting Frame:** Prevents you from placing markers anywhere but inside the Counting Frame.
- **Restrict Placing Markers To Acceptance Area:** Prevents you from placing markers anywhere but within the acceptance area.
- **Restrict Placing Markers To Range of Optical Disector:** Prevents you from placing markers anywhere but within the range of the Optical Disector.

AutoMove

AutoMove is an automatic centering procedure for use with motorized stages. It acts in conjunction with a dashed rectangular boundary, known as the AutoMove Area to allow for the continuous tracing of structures

Automatic Movements

- **Enable Automatic Moves To Top Of Section:** Enables a move to the top of the section when moving to a new sampling site.
- **Enable Automatic Moves To Top Of Disector:** Enables a move to the top of the disector (3D counting frame) after the top of the section has been marked.

Other Options:

- **Disable The Automove Box During Stereological Procedures:**Removes display of the AutoMove box when a stereological procedure is started.
- **Automatic Focus Scanning Rate:** Set the rate of scan if automatic focus scanning is used.

Cavalieri

The Cavalieri tab lets you set options for the Cavalieri probe.

Options

- **Allow Snap To Grid for Cavalieri Probe:** Snap to grid mode enables the placement of markers over the nearest grid intersection. In snap to grid mode, modes such as Paint and Marquee are also available.
- **Show Cavalieri Grid In Show Whole File Mode:** When the entire file is shown, the Cavalieri grid is superimposed on the tracing.
- **Display Cavalieri Grid Using Circles:** With this option selected, circles are drawn around every fourth cavalieri point.

Results

The **Results** tab lets you set various options related to the display of your results.

General Settings

Expand All Calculations When Viewing Results: When this option is selected, the contents of the Sampling Results box are shown fully expanded.

Display Partial Calculations of Formulas: This option controls the display of the planimetric variance details in an **SRS** probe; when checked, displays the covariogram values (A, B and C).

Fill Bottom Entries of Columns With Zeroes: If this option is not selected, columns with no recorded values are blank.

Sort Sections In Ascending Order: Sorts sections in the display of probe runs.

Volume Estimations

Display Planimetric Results: Displays planimetric calculations of area and volume of each contour.

CE Selections

These options allow you to control which CE values are displayed.

- **Display Gundersen CE, Display Schaeffer CE,** and **Display Schmitz-Hof CE:** Controls the display of these results.

- **Only Display Gundersen CE If 3 or More Runs Exist:** Since the Gundersen CE takes into account values from neighboring sections, it is only valid if 3 or more runs have been performed. This option keeps invalid Gundersen CE values from being displayed.

It may be helpful to turn this option off if you are running a pilot study and want a rough idea of what the CE value will be.

Other Options

Display Raw Data Report: The raw data report includes the number of counts for each sampling site in each probe run.

Display Section Order Report: The section order report lists all sections in order of their actual Z position.

Number of Z histogram bins: Use this option to control the number of bins for the Z histogram report.

Color and Tick Marks

Use this tab to set the Probe colors and the style and size of Tick marks.

Probe Colors

- **Set Probe Color, Set Excited Color:** Click to display the Color dialog box, which you use to change the colors for probes or excited items.

Tick Mark Style

- **Change Tick Mark Style:** Click to change your marker style with the C**hange Marker Type dialog** box.
- **Tick Mark Size:** Type a new pixel size for the markers.

Probes Menu

Use this tab to set options and customize Probes. Only those probes checked here appear on the Probes menu.

Click a box next to a probe name to activate the probe on the Probes menu. Clear the checkbox next to a probe name to remove the probe from the Probes menu.

Display Preferences

This menu option displays a tabbed dialog box. The contents of each tabbed page are listed next. This dialog box is an important hub of the Stereo Investigator program that is used quite frequently as it contains many features for controlling the display of tracing data within Stereo Investigator.

View

Options on this page control how, and what objects are viewed on the display.

Mode

- **Show Current Section Only:** When this option is selected, only the tracing that is associated with the current section is displayed. This allows multiple sections to be in the same tracing file while

only viewing the tracing of one section at a time. You are thus able to work on the current section without distraction or visual obstruction by data from previous sections. When this option is deselected, all the data from the other sections as well as the data from the current section is seen together.

This feature is also useful when editing previously traced data. Use this feature in conjunction with **Show Suppressed As Gray** to quickly step though a data set and see individual sections displayed independently from the rest of the tracing. The selected section is shown in color while data belonging to all other sections is shown in gray. Tracing data can be deleted, moved, or otherwise modified in the selected section.

- **Show Suppressed as Gray:** This works in conjunction with **Show Current Section Only**. It shows the suppressed contours and markers (i.e., those that belong to sections other than the currently selected one) in gray.

This feature is very useful for visualizing and editing data. It allows the currently selected section to be displayed in the context of the entire tracing

Line Rendering Attributes

- **Display Thickness:** Displays the thickness of the lines used to trace contours or trees. Line thickness is defined while tracing according to the size of the circular cursor (diameter). Use this feature to view the actual thickness of the lines that are traced.

It is important to understand that the term thickness is used because that is what is seen. The lines produced in the tracing are not lines, but actually three dimensional cylinders and frusta. A frustum is a section of a cone, like a cylinder, but tapered. The tracing created in Stereo Investigator is an exact 3-dimensional representation of the object being traced. When the 3-dimensional tracing is displayed on the flat screen the result is a thick line if thickness is turned on.

Line thickness can be controlled with **Options>General Preferences>Cursor>Circular Cursor Size**, the plus and minus keys on the numeric keyboard, or with the mouse wheel.

The thickness of traced lines (as controlled by the circular cursor size) is always recorded. Thickness affects only the display of the line thickness. It is often more convenient to have this attribute turned off, as thin lines obscure less of the object being traced.

You can select one of four rendering methods:

- Tapered Thickness
- Next Point's Thickness
- Thickness Ratio (Larger)
- Thickness Ratio (Smaller)

Colors

- **Color:** Sets the display mode to color. When this is enabled, objects are drawn using the colors selected by the user.
- **Monochrome:** Sets the display mode to monochrome. This mode should be used in conjunction with the monochrome Lucivid. It is possible to switch back to color for editing and visualization

when using the computer monitor. Some of the features displayed in the tracing window may be displayed slightly differently in monochrome mode. The differences in display make up for information that is displayed using color.

- **Dim Monochrome:** Sets the display mode to dim monochrome. Use this feature when the bright monochrome display overpowers your image. This is useful when using the Lucivid. It provides for computer graphics superposition on the microscope image. to trace very dim structures, such as weak fluorescent stains.

Reference Point and AutoMove Box

Controls the color of the reference point and line surrounding the **AutoMove** box. Click **Set Color** to select a new color. You can change the reference point radius by entering a different number. All subsequently mapped data will be based upon this origin. Choose an easily identified point not far from the area you will be studying so that you can return to it without difficulty. If you are working with serial sections, the reference point is best located near the initial section of the series.

The **Radius** box setting is in pixels, so that the reference point is the same size regardless of the lens being used.

Tracing Transparency

You can set the amount of transparency (or opacity) for tracings. Move the slider to the desired setting.

Accessories

This group of options controls the display of various toolbars, windows, and utilities.

General

- **Focus Position Meter:** This enables the display of the **Focus Position Meter,** providing a visual indication of the current focal depth. The **Focus Position Meter** displays the Z coordinate at the top of the meter. This value always matches the Z coordinate displayed in the lower left corner of the main window in the status bar. The focus position meter is resizable and can be changed in size by dragging the borders of the window. The focus position meter is also used to display the range of a depth filter with a green bar along the left side of the meter.
- **Contour Measurements:** This controls display of the **Contour Measurements** window.
- **Macro View:** This controls the display of the **Macro View** window, in which an aerial view of the tracing and its current field-of-view, can be seen. This is convenient for observing where you are while performing an automated scan, or while tracing a large contour.
- **Marker Summary:** When this option is selected the total number of each marker type traced is displayed on the marker toolbar. You can also right-click on the **Marker** toolbar and choose **Show Marker Summary.** The totals change as markers are traced, deleted, or changed from one type to another. The marker summary is useful for immediate feedback on populations and relative numbers. See Neurolucida Explorer analyses for more information about markers and the various ways to tally populations.
- **Marker Names:** Displays the name of a marker when the marker is displayed.
- **Orthogonal View:** This controls the display of the Orthogonal View window, in which a "side view" of the tracing can be seen.

Center Mark

Displays a special marker at the center of the image. For optical lenses, this is the center of the screen. For video images, this is the center of the video image. For images from bitmapped files, this is the center of the bitmap, or the center of the currently selected bitmap if multiple images are loaded. Click **Set Color** to select a different color. To avoid confusion, you may want to set the center mark to be a different color than the reference point. The center mark for optical lenses is approximately at the optical axis for these lens types. The goal is to position the Lucivid so that the center mark coincides with the optical axis of the lens. The parcentric calibration correction is minimized when the center mark coincides with the optical axis. The same is true with a video lens.

Toolbars

These options are used to enable and disable the various toolbars. If a toolbar is accidentally deleted, these options can be used to restore it.

We recommend that the Status Bar is always enabled, even if you are working with all other toolbars disabled, as the Status Bar contains valuable information about what the program is expecting you to do next. In addition, the Main toolbar options for selecting contour, process and ending types are not contained in any menu items, so this toolbar is necessary for tracing.

Grid

The Grid is a rectangular overlay that may be displayed for a number of uses. It can be used for checking the calibration of lenses. It is also a convenient method of dividing a region into fixed areas for further analysis. The grid is anchored at the reference point and moves along with the tracing as the tracing and stage are moved.

Grid Enabled

Displays a rectangular grid whose size is defined by Grid Spacing.

Bright Grid

Causes the grid to be displayed at maximum brightness. It promotes easier grid visibility when the slide illumination levels are high. Normally, the grid is shown at medium brightness.

Grid On When Whole File Shown

Determines whether the grid is displayed when an aerial viewing mode is selected. Disable this option if the grid display is too dense when using **Go To**, or **Where Is**.

Show Grid Labels for Each Cell

Show Grid Labels shows the coordinates of the grid, with A0 being the coordinate of the reference point, with letters going up to the right of the reference point, and numbers increasing below the reference point. Grid intersections to the left of the reference point are listed with negative letters; intersections above the reference point are listed with negative numbers.

Grid Spacing

This permits you to set the dimensions of the grid. When you change the objective from one magnification to another, the grid changes size accordingly. The grid is not displayed if the spacing is too dense. At low magnifications the grid can become so dense that the entire screen is covered with nothing

but grid lines. Stereo Investigator prevents this by automatically turning off the grid when the spacing between grid lines drops below 4 pixels.

Markers

This page provides control over the display of markers.

Most of these functions are also available when you right-click on the Marker toolbar.

Marker Sizing

Selects the size to display the markers.

- **In Pixels:** Displays all markers at the specified pixel size. Changing the lens has no effect on the display size of the markers. This is the usual method of displaying markers.
- **In Microns:** Displays all markers at the specified micron size. The size of the markers depends on which lens is selected. The markers are scaled along with the tracing. A marker is guaranteed to be displayed at least 1 pixel in size. Markers do not disappear, but are scaled as small as possible.
- **Intrinsic:** Displays each marker according to its intrinsic size. The intrinsic size for each marker is defined by the diameter of the circular cursor when the marker was traced. Since this size is defined in actual micron values, the displayed size depends on which lens is selected. A marker is guaranteed to be at least 1 pixel in size. If a low power lens is selected, markers do not disappear, but are scaled as small as possible. The intrinsic setting allows each individual marker to be displayed at a different size.

Marker list box

- **Marker Name:** Each marker is originally assigned a default name. To assign a different name to a marker, highlight the current name and type in the new one. The name of the marker is used in all subsequent reports. For example, if three markers are renamed to *neuron, glial cell* and *blood vessel*, instead of seeing the number of Marker 1, Marker 2, etc. in a report, the report states the number of neurons, glial cells, blood vessels, etc.
- **Hidden:** The **Hidden** check box lets you turn on and off the display of all markers of that type. These check boxes are located to the right of each marker name

Click **Edit>Reveal Hidden Objects** to view all hidden objects. You can then select individual markers by right-clicking and selecting **Restore Selected Hidden**.

All Visible Button

This turns off the hidden attribute for all marker types. All hidden markers are displayed.

None Visible Button

This makes all markers hidden.

Set Color Button

This button allows you to change the color used to display the markers of the selected type. You can also double click on the color box to the left of the marker name to change the color. The color selection dialog box makes it easy to change the color of the markers to any of the basic system colors or to any color available on your system.

Default Colors Button

This button is used to restore the default colors for each marker type.

Contours

This page provides control over the display of contours.

You can enter very long contour names, but names longer than 12 characters are not completely visible in the contour selection list on the Main toolbar.

Add Contour Type

Adds a new contour to the Contour list box.

Markers list box

- **Default Colors Button:** Resets the colors assigned to each contour to their default setting.
- **Hidden:** To the right of each contour name is a Hidden check box, which determines if contours of that type are displayed. If the box is checked, the contour is not visible, i.e., it is hidden.
- **Contour Name:** Each contour type is assigned a default name. Stereo Investigator allows you to tailor the names of the contours to your preference. Choose specific contour names that are informative for identifying regions you are mapping. The name of a contour may be changed by using the cursor to highlight an existing name and then typing in the new name.

  When a contour type is marked as hidden, its entry is removed from the contour name list of the Main toolbar, so contours of this type cannot be drawn. If a contour of the type being marked as hidden is currently being drawn, the portion that has been traced becomes invisible and the remainder, traced after the contour type is marked hidden, becomes invisible with the next screen refresh.

The hidden check box for each contour type turns on and off the display of all contours of that type. These check boxes are located to the right of each contour name. Hidden contour types are not shown on, and cannot be selected from, the contour name list of the Main toolbar.

Click **Edit>Reveal Hidden Objects** to view all hidden objects. You can then select individual markers by right-clicking and selecting **Restore Selected Hidden Contours**.

All Visible Button

Makes all of the contours visible in the display. All contours that have been hidden become visible.

None Visible Button

Makes all contours hidden.

Set Color Button

To the left of each contour name is a box containing the display color selected for the contour. The contour color is changed by double clicking on the box. The colors dialog makes it simple to select a new color.

Default Colors

Sets the colors to the installed default.

Text

Settings

Choose to display text or represent it as a marker, and set the size of the text marker in pixels.

Default Font

Click **Set Default Font** to display a dialog box where you can change the default font.

Coordinates

Allows you to control which components of the cursor are to be reported. The X,Y,Z coordinates are always reported in the status bar. This page allows the display of the cursor circle diameter and the length of the crosshairs to be enabled or disabled.

Configure Tool Panels

With Stereo Investigator, you can create your own tool panels which contain groups of tools you use most. For example, you might want to have quick access to the **Orthogonal View, Macroview**, and the **Image Organizer**. You could set the three tools as part of a tool panel, which would be displayed when any one of the other tools are displayed. For example, the figure below shows the three tools. Notice that **Orthogonal View** and **Image Organizer** have Minus signs (-) signs in their title bars. This indicates that each tool is "rolled-up". Click on the title bar and the tool opens in the tool panel. Similarly, click on a title bar with a minus sign, and the tool rolls up to just its title bar.

To add a tool to a tool panel

1. Click **Tools>Configure Tool Panels**. Stereo Investigator displays the **Configure Tool Panels** dialog box.
2. Click on a tool.
3. Click on an entry under **Hosted as**.
4. Click **OK**.

Reset_Toolbars

Use this operation to restore all toolbars to their default locations around the tracing window.

Large_Icons

Select this command to switch between large and small icons. This command also redocks all toolbars.

User Profiles

The **User Profiles** command lets you create groups and users, so that you can share settings and lens information. For more information see, Using the profile manager with multiple users.

About Windows and User Profiles: Don't confuse the Stereo Investigator Profile manager with a Windows login or account management function. The information you use to log on to Windows, use for your Internet account, and email are not necessarily the same as the group or profile information you use with User Profiles.

Help Menu

Contents

Opens the **Help** window and displays the **Help Contents** pane, an organized list of topics you can browse.

Index

Opens the **Help** window and displays the **Help Index** pane. This pane contains the keywords we've associated with each topic.

Stereological Formulas

Displays the stereological formulas used by Stereo Investigator.

Tutorials

Use the **Tutorials** to help you learn how to use Stereo Investigator.

Visit Online FAQ

Click this link to visit the MBF Bioscience Online FAQ (Frequently Asked Questions) resource. The online FAQ contains questions, answers, and important information for Stereo Investigator users, and is updated regularly. You must be connected to the Internet to use this command.

Live Support

Click to start an online Live Support session with MBF Bioscience support technicians.

Visit Online Knowledge Base

Click this link to visit the MBF Bioscience Online Knowledge Base, a part of MBF Bioscience Support. The Knowledge Base contains questions, tips, and other information to help you get the best use out of . You must have Internet access and an account with MBF Bioscience Support to use this resource.

Authorize License

Select this option to access the Feature Authorization window. For more information, please see.

System Settings

Click to view information about Stereo Investigator and your system setup.

This information is useful when working with MBF Bioscience Support to diagnose problems.

Diagnostics Window

Click to display the **Diagnostic Window,** which displays operational information including internal error messages, error or information messages from hardware (when available) and diagnostic information.

The information from the **Diagnostic Window** is used by MBF Bioscience Support to help diagnose and solve hardware and software issues.

About Stereo Investigator

This command displays information about Stereo Investigator including version number, licensing, build dates, and other information. You can also use links in this dialog box to visit the MBF Bioscience website for product support, and to download the latest version of Stereo Investigator

Chapter 20

Keyboard Shortcuts

Stereo Investigator contains keyboard shortcuts to speed your work. The keyboard shortcuts that are described here refer to a standard U.S. keyboard layout. Keys on other layouts might not correspond exactly to the keys on a U.S. keyboard.

What if I'm using a special or non-standard keyboard?: If your keyboard has the Ctrl, Alt, and Shift keys, you'll be able to use these shortcuts. If your keyboard or keyboard software lets you redefine key combinations or assign actions to Function or other "macro" keys, you should make a note of any keyboard shortcuts you've redefined or changed.

For keyboard shortcuts in which you press two or more keys simultaneously, the keys to press are separated by a plus sign (+). For keyboard shortcuts in which you press one key immediately followed by another key, the keys to press are separated by a comma (,).

Menu command keys

Each menu has an accelerator key, shown as an underlined letter in the menu. For example, the File menu accelerator is F, the Options menu accelerator is O, and so on. Within each menu, many of the commands also have accelerators, also shown as an underlined letter. For example, to start a new data file, the keyboard shortcut is Alt+F, Alt+N. Some commands may have more than one keyboard shortcut. Use the shortcut that makes sense to you.

To do this...	...press this
Save a file with a new name	CTRL+A
Blackout or restore the interface	CTRL+B
Start a new file	CTRL+N
Open an existing data file	CTRL+O
Print the current file	CTRL+P
Save the current file	CTRL+S
Grab the current video display	CTRL+G
Toggle live video	CTRL+L
Autofocus	CTRL+F
Acquire an Image Stack	CTRL+H

Stereo Investigator tracing keys

Use these keys when performing manual tracing.

To do this...	...press this
Hide/show the tracing	CTRL+T
Add a node	CTRL+ALT+A
Add a tree ending	CTRL+ALT+E
Add a spine to a tree	CTRL+ALT+S
Add a trifurcating node	CTRL+ALT+T
Place a varicosity	CTRL+ALT+V

Editing keys

Use these keys when editing.

To do this...	...press this
Delete the selection	DEL or CTRL+X
Cut the selection to the Windows Clipboard	SHIFT+DEL
Paste the contents of the Windows Clipboard	CTRL+V or SHIFT+INS
Copy the selection	CTRL+C or CTRL+INS
Undo the last edit action	CTRL+U
Save the current file	CTRL+S
Grab the current video display	CTRL+G
Toggle live video	CTRL+L
Autofocus	CTRL+F
Acquire an Image Stack	CTRL+H

Imaging and image stacks keys

Use these keys when working with images.

To do this...	...press this
Big nudge image left	SHIFT+Left arrow
Big nudge image right	SHIFT+Right arrow
Big nudge image up	SHIFT+Up arrow
Big nudge image down	SHIFT+down arrow
Toggle viewing images	0 (zero on keypad)
Zoom in 2x on an image	CTRL++ (plus on keypad)
Zoom out 2x on an image	CTRL+- (minus on keypad)

To do this...	...press this
Display first image in the stack	HOME
Display last image in the stack	END
Display next image in the stack	Page Up
Display the previous image in the stack	Page Down
Delete the image stack plane	ALT+U
Display Min projection	CTRL+SHIFT+M
Display Max projection	CTRL+M

Image filters keys

Use these keys to control the use of color filters.

To do this...	...press this
Display image with no filter	F9
Display image with Red filter	F10
Display image with Green Filter	F11
Display image with Blue filter	F12

Cursor keys

These keys control the crosshair cursor and circular pointer.

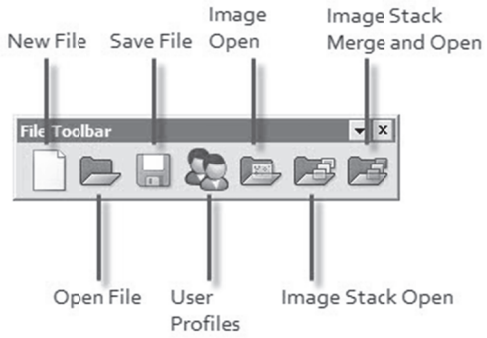
To do this...	...press this
Increase crosshair size	CTRL+Up arrow
Decrease crosshair size	CTRL+Down arrow
Make crosshair thinner	CTRL+Left arrow
Make crosshair thicker	CTRL+Right arrow

Toolbars

The Toolbars contain shortcut buttons for the features most commonly used in Stereo Investigator.

The File Toolbar

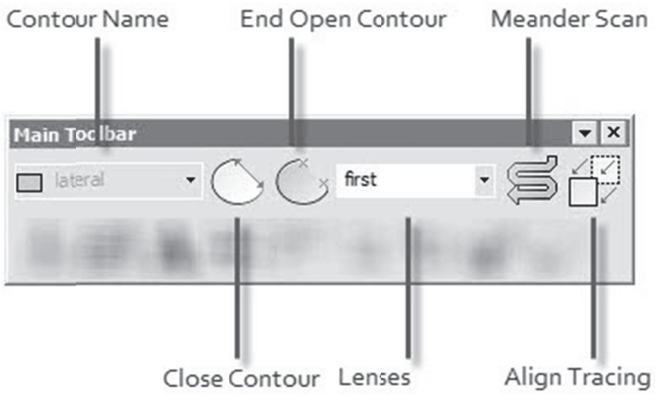
Items in the File toolbar correspond to commands in the File menu, with the exception of the User Profiles button from the Options menu.



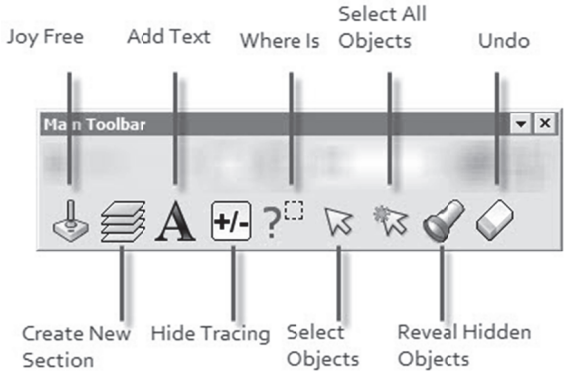
- **New File** lets you start a new data file. If an existing data file is open, Stereo Investigator asks if you want to save it before starting a new one.

- **Open File** lets you open an existing data file If an existing data file is open, Stereo Investigator asks if you want to save it before starting a new one.
- **Save File** will save the data file.
- **User Profiles** opens the **Profile Manager**, which you can use to save settings for individual users and groups, making it easier to share Stereo Investigator.
- **Image Open** opens an existing image file.
- **Image Stack Open** is used to open an image stack.
- **Image Stack Merge and Open** will open and merge an image stack.

The Main Toolbar



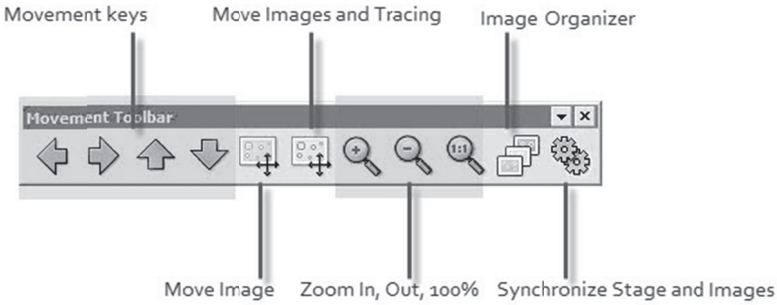
- **Contour Name** lets you choose a name for the contour.
- **Manual Tracing** places you in manual tracing mode.
- **AutoNeuron** starts the **AutoNeuron** workflow.
- **Contour Type** lets you select the drawing type for the contour. You can select from **Freehand, Ribbon, Circle,** or **Square**.
- **Close Contour** closes the open contour.
- **End Open Contour** ends the contour, leaving it open.
- **Lenses** lets you select from the installed lenses.
- **Meander Scan** stars a meander scan operation.
- **Align Tracing** starts the operation to align the tracing with the image.



- **Joy Free** toggles **Joy Free** mode.
- **Create New Section** starts the **Serial Section Set Up** operation.
- **Add Text** lets you add text to your data file.
- **Hide Tracing** hides the tracing, making it easier to see the image.
- **Where Is** enters **Where Is** mode.
- **Select Objects** opens the **Edit Tool** panel, which you use to select objects for editing, slicing, or detaching.
- **Select All Objects** selects all objects on screen.
- **Reveal Hidden Objects** shows any objects you've hidden.
- **Undo** reverses many actions.

The Movement Toolbar

The **Movement** toolbar controls movement, display size, and some image functions.

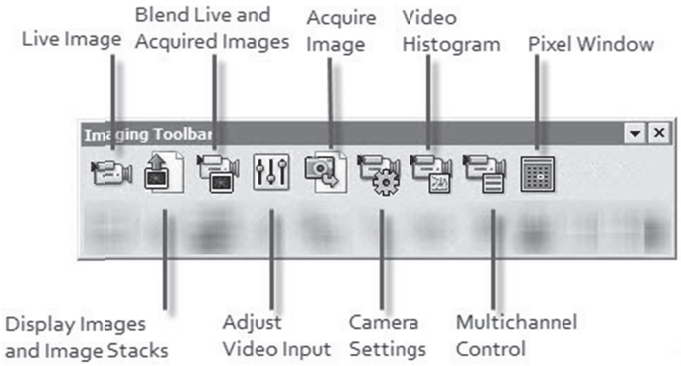


- **Movement keys** are used to move the tracing one screen in the selected direction.
- **Move Image** lets you grab and drag the image to a new location.
- **Move Images and Tracing** lets you grab and drag the image and tracing to a new location.
- **Zoom In, Zoom Out, 100%** zooms the display in the desired direction.
- **Image Organizer** display the Image Organizer Tools panel.

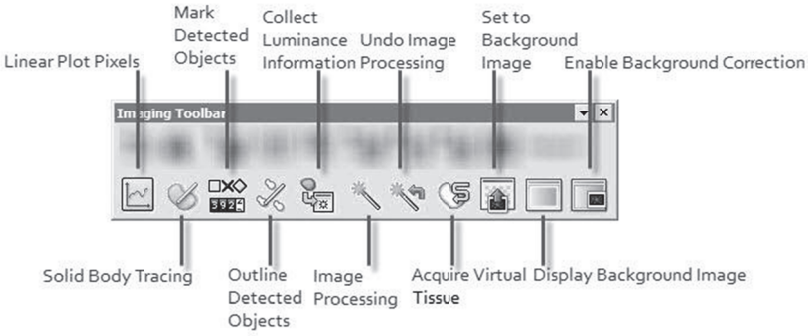
- **Synchronize Stage and Images** synchronizes the state position with the image motion.

The Imaging Toolbar

The **Imaging** toolbar buttons control image acquisition, coloring, and other image manipulation functions.

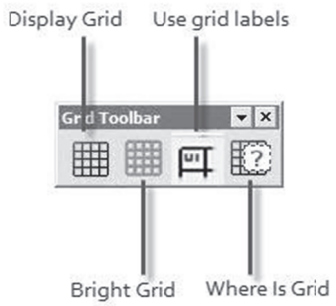


- **Live Image** displays the live image.
- **Display Images and Image Stack** displays the images and image stacks.
- **Blend Live and Acquired Images** blends both the live input and acquired images on screen.
- **Adjust Video Input** is used to adjust your video input.
- **Acquire Image** acquires and displays an image from the camera.
- **Camera Settings** displays the **Camera Settings Tools** panel, used to adjust your camera settings.
- **Video Histogram** displays the **Video Histogram Tools** panel, used to adjust and modify video settings.
- **Multichannel Control** displays the **Multichannel Control Tools** panel, used to direct and control hardware used in multichannel acquires.
- **Pixel Window** displays the **Pixel Luminance** window, which displays a value for pixels under the mouse pointer.



- **Linear Plot Pixel** displays the **Linear Pixel Plot** window, which displays, a graph of the pixel luminances along a line segment. This window provides a graphical version of the information displayed in the pixel window.
- **Solid Body Tracing** starts **the Solid Body Tracing** function. Click inside a solid body to be autotraced.
- **Mark Detected Objects** marks detected objects
- **Outline Detected Objects** outlines detected objects.
- **Collect Luminance Information** collects the luminance information of a closed contour, and displays this information in the **Contour Measurements** window.
- **Image Processing** opens the **Image Processing** dialog box, which can be used to modify the image.
- **Undo Image Processing** reverses the image processing changes.
- **Acquire Virtual Tissue** starts the Virtual Tissue acquisition function.
- **Set to Background Image** displays the **Correction Type** dialog box, used to set the background to brightfield or fluorescent.
- **Display Background Image** displays the acquired background image.
- **Enable Background Correction** sets background levels based on the background image.

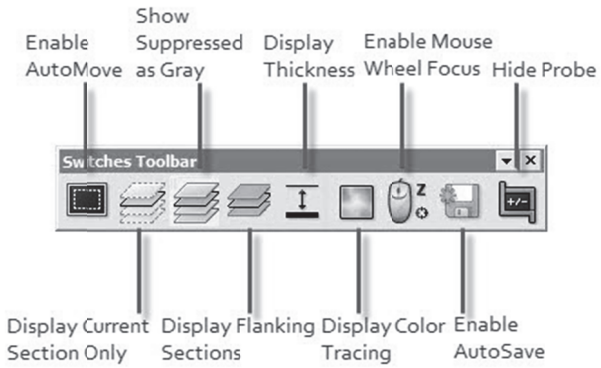
The Grid Toolbar



- **Display Grid** toggles the display of the grid.
- **Bright Grid** displays a brighter grid.
- **Use grid labels** displays the grid with coordinate labels, starting at the reference points, which is grid square A0.
- **Where is Grid** displays the grid in Where Is mode.

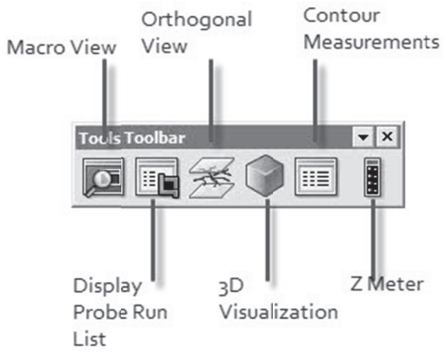
The Switches Toolbar

The Switches toolbar controls many display control features and program functions that can be toggled on and off.



- **Enable AutoMove** enables or disable **AutoMove**.
- **Display Current Section** Only displays just the currently active selection.
- **Show Suppressed as Gray** shows the suppressed sections as gray, making the unsuppressed section easier to see.
- **Display Flanking Sections** displays any sections flaking the current section.
- **Display Thickness** will display your tracings as thick as you have drawn them.
- **Display Color Tracing** displays tracings in the color you have assigned.
- **Enable Mouse Wheel Focus** lets you use the mouse wheel to focus.
- **Enable Auto Save** automatically saves the tracing, determined by the setting in **Options>General Preferences>AutoSave** tab.
- **Enable Combined Markers** toggles combined markers, useful when marking an area stained with 2 or more methods.
- **Hide Probe** hides the probe on screen

The Tools Toolbar

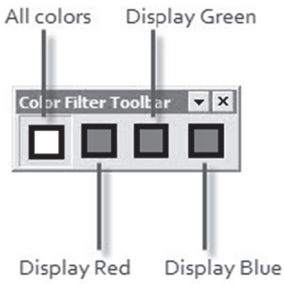


- **Macro View** toggles the display of the **Macro View** tools panel.
- **Display Probe Run List** displays information about all the probe runs
- **Orthogonal View** toggles the display of the **Orthogonal View** tools panel.

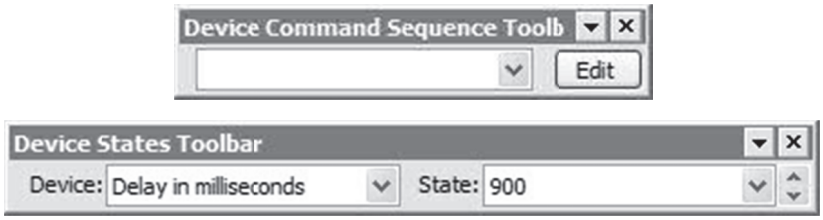
- **3D Visualization** toggles the display of the **3D Visualization** tools panel, which you use to see a 3D representation of your data and tracings.
- **Contour Measurements** toggles the display of the **Contour Measurements** tools panel, which displays information about each contour.
- **Z Meter** toggles the display of the **Z Meter**, which shows the current Z position.

Color Filters Toolbar

Click a button to display all color channels or only one selected channel.



Device Command Sequence and Device States Toolbars



Both of these toolbars support the integration of Olympus microscopes controlled by the IX2-UCB or BX2-UCB with DSU attachments.

Device Command Sequence

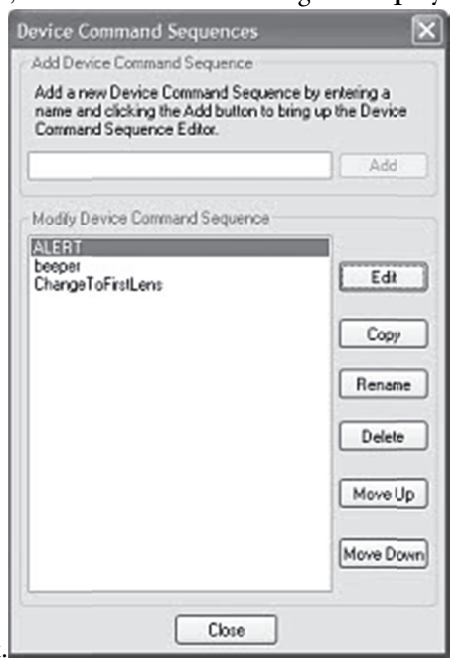
The Device Command Sequence toolbar lets you select the specific command sequence or sequences you want Stereo Investigator to execute. You can also edit or create new command sequences.

To execute a command sequence

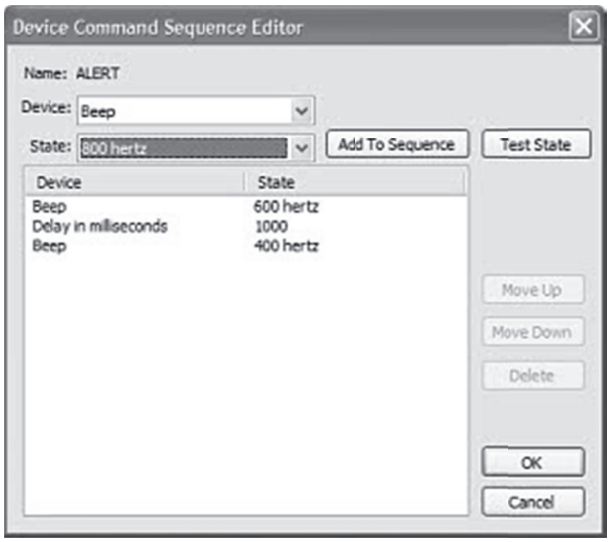
- Select an existing command sequence from the drop-down list box. Stereo Investigator begins execution of the sequence.

To edit a command sequence

On the **Device Command Sequence** toolbar, click Edit. Stereo Investigator **displays** the **Device Command Sequences** dialog box.



1. Select a sequence under **Modify Device Command Sequence** and click **Edit**. Stereo Investigator displays the Device Command Sequence Editor dialog box.



2. Select the device and then select the state. Click **Add to Sequence**. The device and state appear at the end of the Device/State list. You can also click **Test State** to see what happens when Stereo Investigator executes the device/state pair.
3. Use the **Move Up** and **Move Down** buttons to rearrange the items in the list. Use the **Delete** button to remove a device.
4. Click **OK** when complete.

Device States

You use these drop-down list boxes to select or view a Device and then a State for a device that can be controlled by a Device Command Sequence.

The default devices shipped with Stereo Investigator are:

- Beep: 100 hertz - 50000 hertz
- Delay in Milliseconds: Pause to 30000 milliseconds
- Lens: Any of the already defined lenses on your system
- Message: The name of the message you have defined

In addition, any equipment you add or attach to your Stereo Investigator installation may contain devices that can be controlled by a Device Command Sequence. For example, the Olympus BX51 and Olympus B61 microscopes with DSU (Disk Spinning Units) attachments allow a sophisticated level of control of the microscopes and DSU.

Click the **Auto Thickness on/off** button to toggle Auto Thickness.

Type a value or use the control arrows to set the threshold for automatic thickness.

Index

3

3D, 241
3D Reconstruction, 231

A

Account for Missing Sections, 208
Acquire Image, 256
Acquire Image Stack, 256
Acquire Multichannel Image, 256
Acquire Setup, 259
Acquire SRS
  Stack or Series, 259
Add Text, 232
Adjust Video Input, 254
Align
  tracings, 238
Aligning
  tracing and specimen, 32
Apotome
  Optical Section Quality, 116
ApoTome, 115
  settings, 115
Area Fraction Fractionator Probe, 159
Automatic Contouring, 241
AutoMove, 34, 35, 240
AutoMove Settings, 34

B

Background Images
  Acquiring, 257

C

Calibration
  data tablet, 23
  focus step size, 27, 247
  imported images, 22, 33
  Macro lens, 22
  Parcentric and Parfocal, 25
camera lucida, 71
Cavalieri Probe, 137, 153, 160, 180
CE Cruz-Orive, 129
CE Gundersen, 128
CE Scheaffer, 128
CE Schmitz-Hof, 129
Center point
  Define, 34
Center Point, 240
Clipboard
  Paste, 231
Coefficient of Error, 119, 127, 138, 142
Coefficient of Error (Gundersen), 128
Collect Luminance Information, 272
Color Channels, 273
Combined Slope Intercept, 121, 187
Connectivity Assay, 140
Contours
  basic measurements, 41
  display properties, 304
  luminance information, 42
coordinate system, 71
Copy to Clipboard
  BMP, 232
  Metafile, 232
Counting Frame, 135
Cycloids for Lv, 121, 126, 191
Cycloids for Sv, 121, 190

D

deconvolve, 269
Deep Focus, 273
Define New Lens, 245
Diagnostics, 307
Discrete Vertical Rotator, 121, 186
Disector, 120, 140, 142, 180, 206
Display Preferences, 299
Display Probe Run List, 210
Docking, 11
Dongle, 7

E

Edit Lens, 247
Enable Background Correction, 258
Error coefficients, 44
Exit, 231
Export Tracing, 222

F

fiducial points, 71
File
  Description, 230
Final Magnification, 248
Force Square, 136
Fractionator, 135, 140, 159, 209

G

General Preferences, 289
Go To, 235, 236
Grid Focus, 115

Grid Tune, 28

H

Hardware
  considerations, 14
Huygens, 269

I

Image Display Adjustment, 265
Image Effects, 283
Image Open, 223
Image Organizer, 253, 254, 282
  Configuring, 305
Image Processing, 267
Image Save, 224
Image Stack
  Merge and Open, 228
  Open, 226
  Save, 229
  Save As, 229
Image stacks, 225, 228
Installing, 14
Interface, 11
Isotropic Fakir Method, 121, 126, 196
IUR Planes Optical Fractionator, 121, 157

J

Joy Free, 31, 238
Joy Track, 31, 236
Joystick, 31, 283

K

Keyboard shortcuts, 309
Kodalith, 271, 279

L

L-Cycloid Optical Fractionator, 121, 155
Lens
  grid tune, 246
  Optical, 23
  Type, 285
  Video, 253
Lenses, 25
  calibrating, 17
Licence
  authorization, 307
License, 7, 307
  finding information, 7
Linear Disector, 206
Linear Optical Disector, 121, 135, 207
Live Image, 256
Lucivid, 15, 23
Luminance
  contours, 42

M

Macroview
  Configuring, 305
Made to Order Layout
  Saving presets, 137
  types, 137
Made to Order Layouts, 136, 179
Markers bar, 11
Match selection, 251
Max Intensity Projection, 273
Meander Scan, 34, 237, 238
Measuring
  quick measure, 242
Merz, 121, 184
Message Device Setup, 287
Microscope Setup, 286
Min Intensity Projection, 273
motorized stages, 14
Mouse shortcuts, 309
Move
  Image, 33
  Image and tracing, 33, 236
Move Image, 33
Move To, 235
Moving around, 31
Moving images, 33
Multiple users, 8

N

Nearest Neighbor Analysis, 162
New Data File, 219

O

Open Data File, 219
Optical Disector, 135
Optical Fractionator Workflow, 140
Optical Rotator, 120, 126, 201
Orthogonal View
  Configuring, 305

P

Parcentric-Parfocal Calibration, 247
Parfocal, 291
Paste
  from Clipboard, 232
photomicrographs, 71
Planar Rotator., 199
Point Sampled Intercept, 188

Population Counts, 119, 140
Population Estimate, 138
position encoders, 14
Preferences
  General, 289
Print, 230
Print Preview, 230
Probe results, 210
Profile Manager, 8

Q

Quick measure commands, 242

R

Recent files, 231
Resample Disector, 215
Resample Oversample, 213
Rotational alignment, 17

S

Save / Save As, 222
Section thickness
  Measuring, 144
Select Acquired SRS Series, 209
Select Objects, 231
Serial Section Manager, 148, 179
Serial Sections
  Opening files, 219
Set Stage Z, 238
Shape Information, 43
Shrinkage correction, 248
Spatially Organized Framework for Imaging (SOFI), 34
Stage Setup, 285
Stage Z
  Set, 238
Step Size
  Common Step Sizes, 27
Stereology Preferences, 136
Support, 307
Surface Weighted Star Volume, 121, 193
Surfactor, 121, 205
Synchronize
  Stage and Images, 237
System Settings, 307

T

The Optical Disector, 120
Tissue Preparation for Stereological Probes, 126
To Reference Point, 235
Tool Panels, 11
  Configuring, 305
Toolbars, 11, 311
Total Variance, 128
traced data, flipping, 80
Tracing
  Kodalith, 279
  solid body, 279
tracing upside-down sections, 79
Tracing window, 11
Transparency, 282
  Tracing, 301

U

Undo, 231

V

Variance Due to Noise, 128, 152
Variance of Systematic Random Sampling, 128
Vertical Spatial Grid, 121, 126, 193
Video Blend, 254
video cards, 15
Video Setup, 286
Virtual Mode, Image Save, 225
Virtual tissue, 257
Visit Online FAQ, 306
Volume Density Population Estimate, 121

W

Weibel, 121, 185