

## 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)		

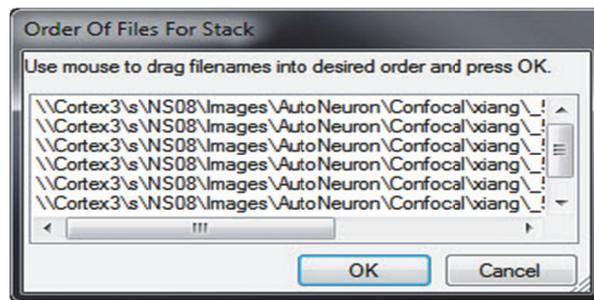
### How do I load image stacks?

---

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.

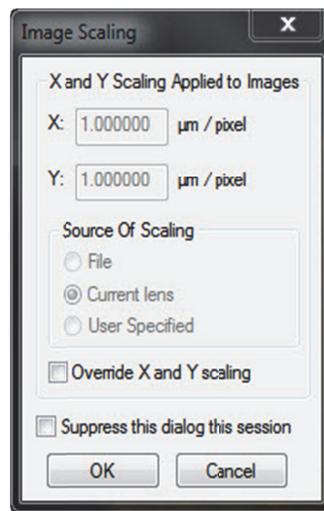
If you select this option, you need to select the correction factor for the physical distance between the lens and the image. Stereo Investigator automatically enter this value for **Air**, **Oil**, and **Water**. If you select **Other**, you must manual enter the factor.

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.

## Stereo Investigator 10 - The Image Stack Module

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.