

GE Healthcare
Life Sciences

DeltaVision™ OMX SR

Customer Instructions
Structured Illumination



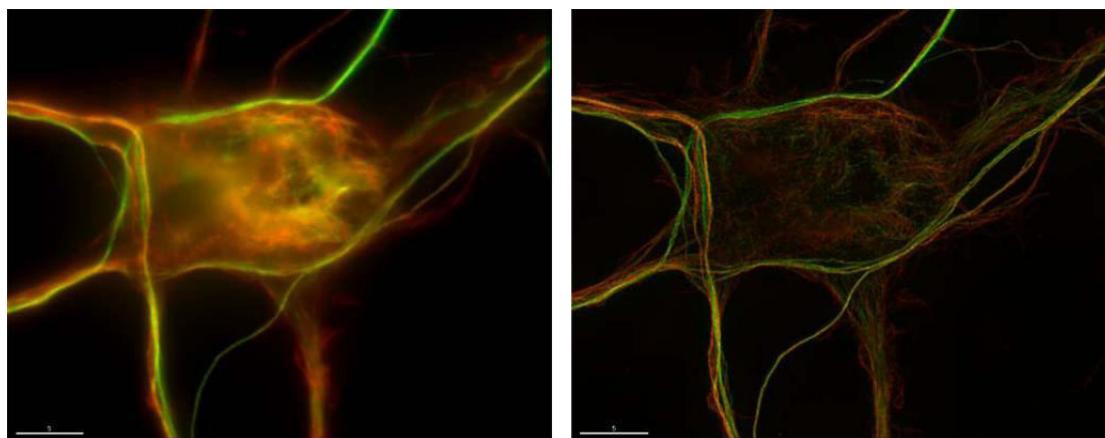
Structured Illumination

- ◆ *Introduces Structured Illumination and how it works*
- ◆ *Describes the three different Structured Illumination options*
- ◆ *Illustrates the methods behind Blaze Structured Illumination*
- ◆ *Provides procedures for performing reconstruction experiments*
- ◆ *Describes how to use the Task Builder for reconstruction experiments*

What is Structured Illumination?

The DeltaVision OMX SR Imaging System is a super-resolution microscopy system that uses structured illumination techniques to surpass previous resolution limits and allows you to image beyond the surface of the coverslip with multiple fluorescent probes. The system's structured illumination technology resolves more of your biological structures and features invisible with traditional microscopy. Using the DeltaVision OMX SR, you can image from one to twenty microns into cells and tissues; and, since the DeltaVision OMX SR works with conventional fluorochromes, there is no need to genetically engineer novel or complex photoswitchable probes. You use the same preparation methods and apply the same fluorescent labeling reagents (antibodies and protein tags) already used in the lab.

Figure 1. Comparison - Standard Widefield (Left) and Structured Illumination (Right)



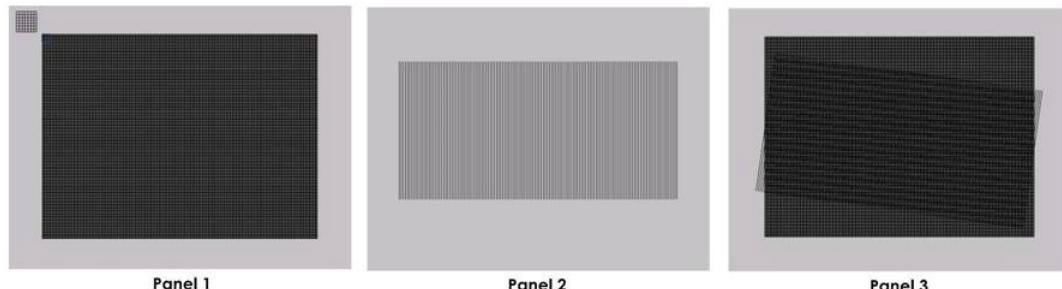
Two isoforms of beta-tubulin in a cultured neuron - Image courtesy of Stephanie Kaech Petrie and Aurelie Snyder, Advanced Light Microscopy Core at The Jungers Institute Oregon Health & Sciences University

How does Structured Illumination work?

Structured Illumination diffracts the laser beam into two or three parallel beams which are combined by the objective to produce 2-D or 3-D interference fringe patterns on the sample. Multiple images of the sample are collected while shifting and rotating the fringe pattern, and stepping through the sample in the vertical direction (Z). The mathematical algorithm at the heart of OMX reconstructs a super-resolution image by solving large systems of linear equations with data from all of the captured images.

The resolution of a microscope is limited by the amount of spatial information that can pass successfully through the optics (commonly referred to as the diffraction limit). In Figure 2, the fine grid pattern shown in Panel 1 represents that spatial information. If this high-resolution information is mixed with a known signal that we can resolve (Panel 2), we generate a moiré pattern seen in Panel 3. The moiré pattern that we see is the difference between the two patterns and can easily be represented without high-resolution methods.

Figure 2. The Moiré Pattern



Similarly, when the DeltaVision OMX SR is in three-dimensional structured illumination mode, the system superimposes a three dimensional illumination pattern onto the sample. This interference pattern generates a moiré pattern that contains information from both the illumination and the biological sample. By carefully reconstructing the sample data from the moiré pattern, we create a super-resolution three-dimensional image of the original sample.

Because the illumination pattern is three-dimensional, the reconstructed image contains both the lateral (2D) and axial (3D) super resolution data. This illumination pattern can be efficiently generated with multiple excitation wavelengths, allowing multiple fluorochromes to be used in the same sample.

Reconstruction Modes on the DeltaVision OMX SR

This section introduces the methods and options for Structured Illumination data acquisition and analysis using the DeltaVision OMX SR system.

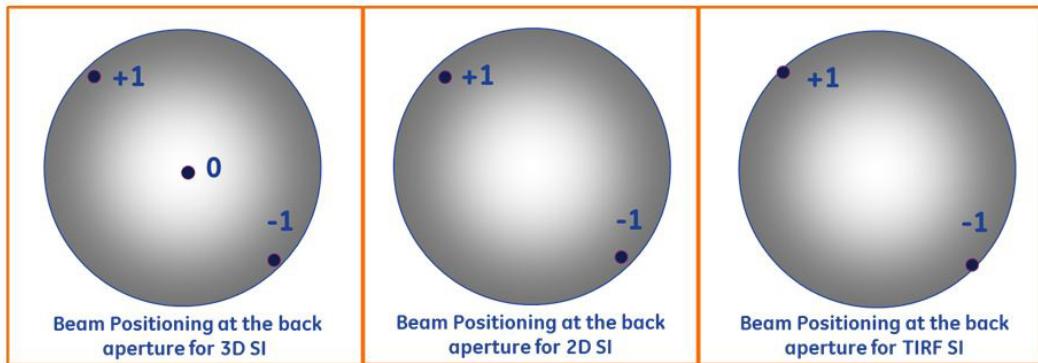
Introduction

The OMX SR is capable of acquiring and processing three types of Structured Illumination data.

- 3D structured illumination (**SI**)
- 2D structured illumination (**2D SI**)
- TIRF structured illumination (**SI TIRF**)

All Structured Illumination patterns are created through the Blaze hardware by positioning the positive and negative first order (+1, -1) beams, with or without the zero order beam, at specific locations at the objective back aperture. Additionally, each excitation wavelength is aligned independently to achieve optimal resolution of all wavelengths.

Figure 3. Beam positioning at the back aperture for 3D SI, 2D SI, and TIRF SI.



Structured Illumination Options

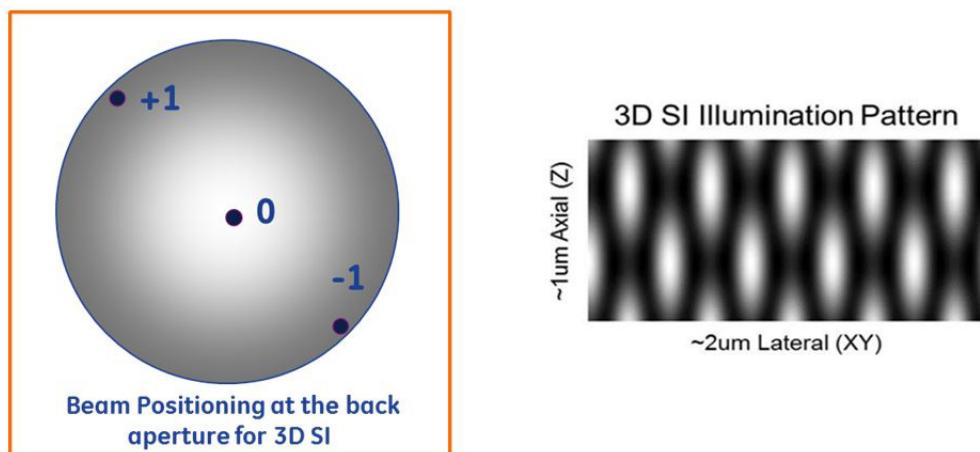
This section describes each of the Structured Illumination options and provides information regarding the benefits and limitations of each option.

3D Structured Illumination (3D SI)

Three-dimensional structured illumination is the most common type of structured illumination and is the technology with which all previous and current OMX systems have been released.

Illumination description:

The +1, -1, and 0 order beams are combined to interfere at the sample plane creating a three dimensional structured illumination pattern.



Images required:

To reconstruct 3D information, each z-section is imaged at 5 phase shifts in 3 angles of rotation for a total of 15 images per z-section. Additionally, at least 8 z-sections spaced 0.125μm apart must be collected for proper reconstruction.

Benefits:

This is the only type of structured illumination that increases resolution along the z-axis of the coverslip. This technique provides a 2X improvement in resolution in the x, y, and z directions. It also provides the best contrast improvement along the z-axis. 3D SI is the standard method of structured illumination. It is recommended for most biological application use cases that include questions requiring more resolution. Many different types of samples have been imaged successfully on the OMX in 3D SI, including fixed and live cellular preparations, microbiological samples, and tissue preparations.

Limitations:

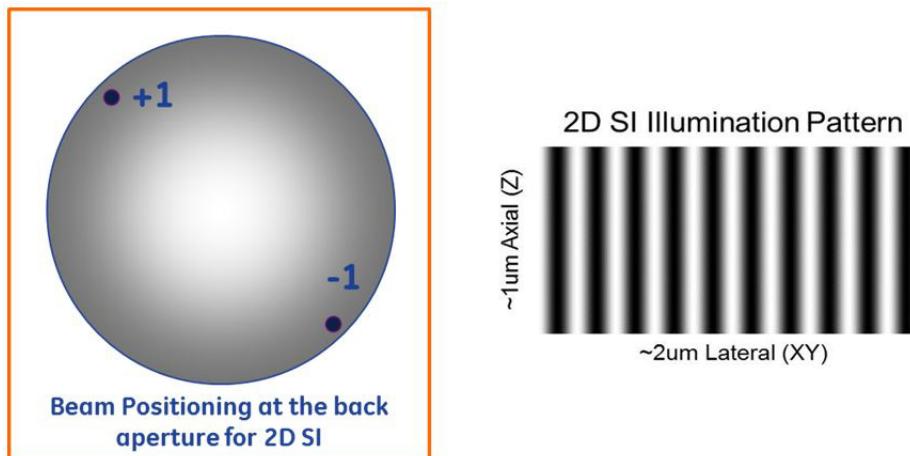
Since 3D SI is a true 3D reconstruction, the minimum number of images required to create a reconstruction can be limiting for certain live samples or samples that bleach quickly. In cases where photobleaching is too significant for successful 3D SI reconstructions or a sample is moving too quickly to capture the 3D SI stack, 2D SI should be used as an alternative to 3D SI.

2D Structured Illumination (2D SI)

Two-dimensional structured illumination is a simpler method of SI imaging which requires fewer images to give a resolution improvement in the x and y directions. There will be no resolution improvement in the z direction, but 2D SI can be performed at any z depth into the sample. If the sample is too challenging for good 3D SI results, either from moving too quickly or bleaching too much, 2D SI is a good alternative.

Illumination description:

The 0 order beam is blocked in the light path and the +1 and -1 order beams are combined to interfere at the sample plane creating a two-dimensional structured illumination pattern that extends through z.



Images required:

To reconstruct 2D information, each z-section is imaged at 3 phase shifts in 3 angles of rotation for a total of 9 images per z-section. Multiple z-sections of data can be acquired; each section is processed independently so the section spacing does not matter.



Note On OMX instruments prior to the DeltaVision OMX SR, 2D SI reconstruction is an option, but hardware limitations require that the 3D SI Interference pattern (5 phase shifts at 3 angles for a total of 15 images per z-section) is used with only 1 z-section selected for acquisition.

Benefits:

This technique provides 2X improvement in resolution in the x and y directions. 2D SI works best on samples that do not have high background or samples where the majority of the information resides in a single z-plane. Fewer images are required for this type of structured illumination, and it can be performed at any depth in the sample.

Limitations:

Since a two-dimensional OTF is used to reconstruct the data, artifacts will be created by out of focus light. This method provides no improvement of z-resolution. If your labeled structures of interest within your sample are directly at the coverslip, SI TIRF could be an alternate 2D imaging option. SI TIRF helps to eliminate out of focus light and subsequent artifacts caused by it in 2D SI reconstructions.

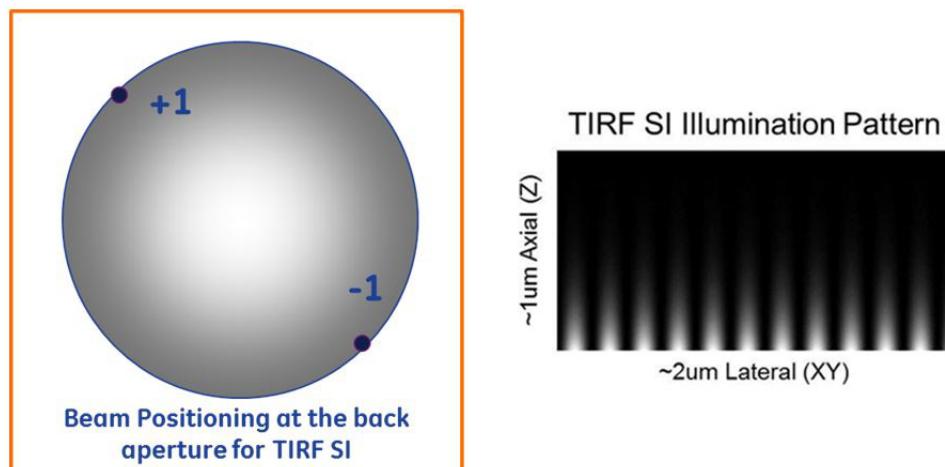
TIRF Structured Illumination (SI TIRF)

TIRF Structured Illumination is a variation of 2D Structured Illumination which produces the structured illumination interference pattern in a Total Internal Reflection mode. This means that, with this illumination option, only samples and structures within the first 250nm adjacent to the coverslip can be imaged.

Illumination description:

The 0 order beam is blocked in the light path and the +1 and -1 order beams are moved out to the edge of the objective back aperture. Both beams create Total Internal Reflectance illumination and interfere with each other to create a structured total internal reflectance illumination pattern. The pattern is essentially the same as the 2D SI pattern

(with a smaller line-spacing), but the SI TIRF pattern decays exponentially along the z-axis, creating illumination solely within the 250nm adjacent to the coverslip.



Images required:

To reconstruct 2D information, each z-section is imaged at 3 phase shifts in 3 angles of rotation for a total of 9 images per z-section. Only 1 z-section of data is acquired, directly at the coverslip within the TIRF illumination plane (<250nm from coverslip).

Benefits:

SI TIRF provides 2X improvement in resolution in the x and y directions. Resolution improvement along the z-axis comes from the Total Internal Reflectance properties of the illumination. This combination of imaging techniques (SI and TIRF) allows three dimensional resolution improvement with fewer images required. Due to the limited depth of the illumination pattern (~250nm), improved contrast can be expected without artifacts from out of focus light.

Limitations:

This type of imaging can only be done at the coverslip interface with aqueous samples. If the sample is directly adjacent to the coverslip and less than 250nm in the z direction, SI TIRF is a good option. If it is anything larger than that or further into the sample, SI TIRF will not work. If this is the case, the 2D SI or 3D SI methods should be used.

OMX SR Blaze Structured Illumination

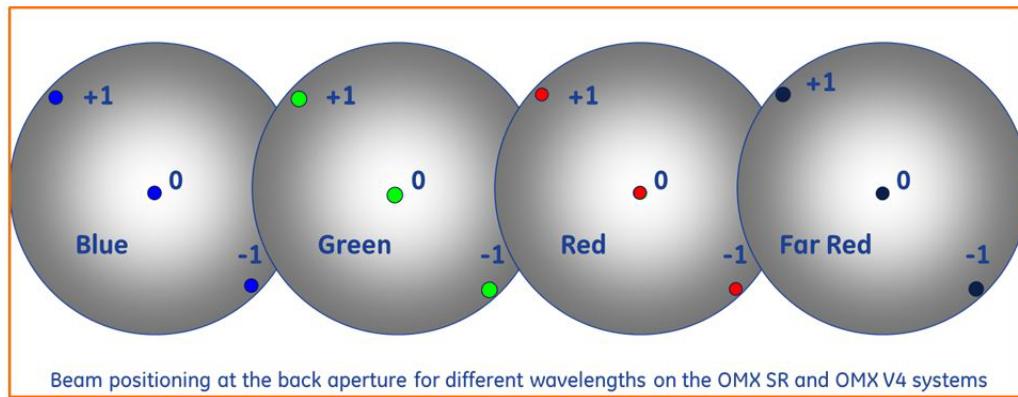
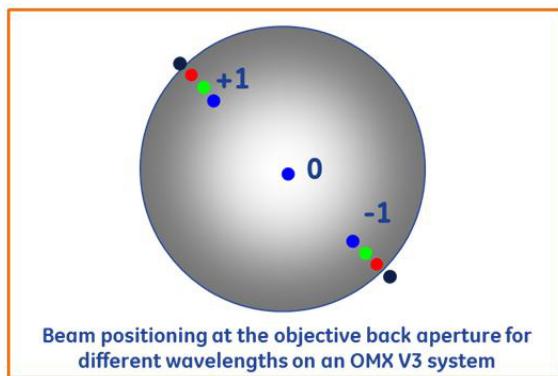
A fiber optic cable directs the laser light into a diffraction grating in the light path which is used to generate multiple orders of the beam. For 3D SI experiments, the zero (0) order beam and the positive and negative first (+1, -1) order beams are conditioned to create the 3D SI interference pattern through the Blaze optical path. For the 2D SI and TIRF SI experiments, the 0 order beam is blocked and only the +1 and -1 order beams are conditioned to create the 2D SI and TIRF SI interference patterns.

Phase Control Module

In all three SI experiment types, optics mounted on galvos are used to manipulate the beams which in turn provide the necessary changes in the illumination pattern to create the desired SI interference pattern. Four galvos are used to control the path length of the

+1 and -1 order beams. A relative change in the path lengths creates the lateral phase shifts of the illumination pattern.

Two additional galvos are used to move the +1 and -1 order beams closer to or further from the center beam, controlling the line-spacing of the illumination pattern. This step in the SI pattern generation is used to achieve the optimal resolution for each wavelength by positioning the +1 and -1 order beams at the edge of the back aperture of the objective. This differs from the original implementation of 3D SI on the OMX V3 instrument, where a compromise had to be made and optimal resolution could only be achieved for the longest wavelength chosen. Most commonly 561nm or 593nm were the longest wavelength selected, which allowed for up to 3 SI channels to be included in any 3D SI data set: 561nm/593nm, 488nm and 405nm. The shorter wavelengths were slightly compromised in the resolution they could achieve. Far Red with 642nm excitation falls outside of the optical path in those systems. Aligning to the 642nm laser resulted in a significant loss in resolution gain for the other 3 channels so was not recommended for SI on the early OMX instruments.



Angle Control Module

Rotation of the illumination pattern is achieved by reflecting the 3 beams off of a galvo controlled mirror that rapidly rotates between 3 clusters of angled mirrors. From there, the beams are sent into the optics block and up to the objective to create the interference pattern at the sample imaging plane.

Running Structured Illumination Experiments

For complete instructions on setting up a DeltaVision OMX SR experiment, including mounting a sample and other parameter guidelines, see Chapter 4 in the *DeltaVision OMX SR Getting Started Guide*.

Oil Selection for SI

The first and critical step to the successful performance of SI experiments is to select the appropriate refractive index immersion oil to optimize spherical aberration for your sample, instrument, and room conditions. Many factors need to be considered when selecting oil. These include coverslip thickness, sample mounting media, room/imaging temperature, excitation wavelength, and depth into the sample. This may seem daunting at first, but there is no need to reinvent the wheel each imaging session. There are several suggested guidelines you can follow provided in the table below. For more detailed instructions on selecting your immersion oil, see Chapter 6 in the *DeltaVision OMX SR Getting Started Guide*.

Figure 4. Example of a completed table for a given DeltaVision OMX SR installed with stable room temperature at 23°C.

DeltaVision OMX SR Immersion Oil Selection Guidelines				
Excitation Wavelength	Imaging at the Coverslip and Room Temperature	Imaging +5μm from Coverslip	Imaging +10μm from Coverslip	Imaging at 37°C
405 nm	1.510	1.512	1.514	
488 nm	1.512	1.514	1.516	
568 nm	1.516	1.518	1.520	
642 nm	1.518	1.520	1.522	
Select the Oil for the longest Wavelength to be imaged in the current sample	For every +5μ imaging into the sample, go up 0.002 or 1 step in the oil kit.			

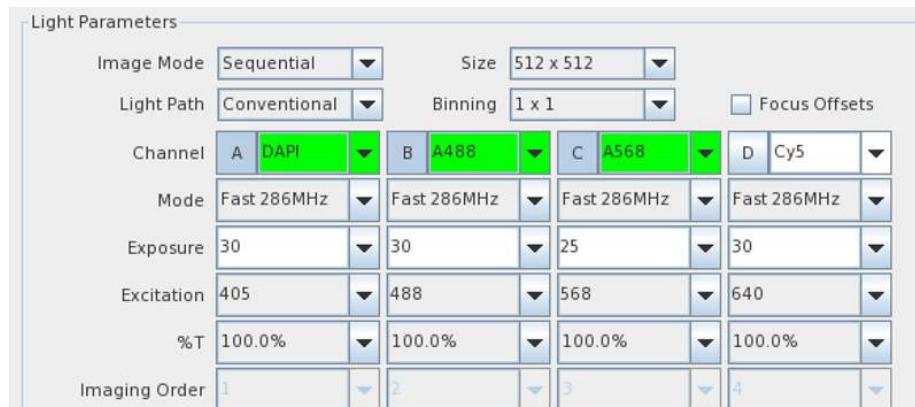
Figure 5. A blank template that can be used to record standard oil selections for a new OMX SR instrument upon installation and training.

DeltaVision OMX SR Immersion Oil Selection Guidelines				
Excitation Wavelength	Imaging at the Coverslip and Room Temperature	Imaging +5um from Coverslip	Imaging +10um from Coverslip	Imaging at 37°C
405 nm				
488 nm				
568 nm				
642 nm				
Select the Oil for the longest Wavelength to be imaged in the current sample		For every +5μ imaging into the sample, go up 0.002 or 1 step in the oil kit.		

To define SI imaging parameters:

1. Ensure all cameras are activated with the correct emission channel and excitation source selected properly. The imaging channel order will follow the selected order.

Figure 6. Select Channel(s)



2. Select the **Light Path**:

- o **SI** for 3D structured illumination imaging.
- o **SI-2D** for 2 dimensional structured illumination imaging
- o **SI-TIRF** for 2 dimensional total internal reflectance structured illumination imaging



Note The Image Mode is set and locked in **Sequential** when any SI light paths are selected. Sequential Mode is the only applicable mode for Blaze illumination. The **Binning** field is locked to **1x1** for all SI experiments.

3. Set the Image Size in the **Size** field to ensure your full sample field of view is captured. (1024x1024 is the maximum image size for the PCO sCMOS cameras.)



Note The illumination area of the structured illumination has been widened to cover the majority of a 1024x1024 field of view. However, due to imperfections of the objective lens, astigmatism worsens farther away from the center of the light path. This will cause image quality and resolution to degrade away from the center 512x512 region.

To determine acquisition parameters:

1. Adjust **Exposure** time and **%T** settings for each channel.
2. Specify the desired thickness of the scan.
 - a. Use the Z-Slider or the Z up and down arrows to move the stage to the top of the image stack.
 - b. Use the **Mark Top** and **Mark Bottom** buttons to mark the thickness. The sample thickness (stack height) you marked is displayed directly below the **Z safety limit** and **Z touchdown** fields.



Note Stack height (marked thickness) is limited to 50 µm, the range of the slide carrier's Z piezo.

To define the experiment:

1. Ensure the **Experiment** tab options are displayed in the main program window and then select the experiment type to run from the drop-down **Type** list.
 - o **SI** for 3D structured illumination imaging.
 - o **SI 2D** for 2 dimensional structured illumination imaging
 - o **SI TIRF** for 2 dimensional total internal reflectance structured illumination imaging



Note If the desired experiment type is not available, ensure the Light Path selected is correct. Available experiment types are determined by the active Light Path.

2. Use the visit buttons to move the stage/sample so that it matches the **Focus point when scan starts** field. The default setting is to start from the middle.
3. Set **Optical section spacing** to the desired thickness value. For SI 3D, this must be 0.125.
4. Set **Sample thickness** by choosing one of the following:
 - o Type in the **Number of optical sections**
or
 - o type in the **Sample thickness**
or
 - o click the **Get thickness** button (if the top and bottom of the sample were previously marked).

5. If a time-lapse experiment is desired, activate the **Time-lapse** check box and then set the parameters as needed.
 - Type the length of time between each time point into the **Time-lapse Hours/ Minutes/Seconds/Msecs** fields.
 - Type the desired number of time points into the **Time points** field. (Note that changing this value automatically updates the **Total time** field, and vice versa.)

or
 - Type the total time for the experiment into the **Total time** field. (As noted above, changing this value automatically updates the **Time points** field.)
 - If you want to collect a subset of your time-lapse data for a particular channel and save it to a separate file, activate the **Subset Time-lapse** check box. Next, in the **Every _ time points** field, enter how often you want to collect the data and activate the **Image** check box for each channel you want the subset of data.

Example: You define a two-channel time-lapse experiment that will collect a total of 10 time points. You activate the **Subset Time-lapse** check box and specify **Every 2 time points** for time-lapse images collected using Channel A. For Channel A, the software collects time-lapse data for time points 1 (time point 1 is always collected during a time-lapse experiment), 3, 5, 7, and 9. The time-lapse data for Channel A is saved separately from the rest of the time-lapse data in a data file named `filename_subset.dv`.)

6. If you want the system to automatically compensate for system axial drift during the experiment, activate either the **HW UltimateFocus** check box or the **SW AutoFocus** check box. Then, set the parameters as needed by clicking the **Settings** button and entering the necessary information.



Note **HW UltimateFocus** is the most commonly used autofocus method and can efficiently compensate for systematic focus shifts (temperature, mechanical, etc) during an experiment. **SW AutoFocus** is less commonly used and can aid in tracking focus differences due to the sample itself (mitotic cell, etc).

HW - UltimateFocus:

- In the **Move threshold (nm)** field, enter the desired maximum measured focus error (in nm) before a corrective action should be taken.
- In the **Maximum iterations** field, enter the maximum measure/move sequences for this action to reach the calibrated focus point.
- In the **Perform every _ time points** field, enter how often to run the UltimateFocus correction routine.

SW - Autofocus:

- Select the **Focus Channel** (A-D) to define which channel is used to determine the best focal plane.
- Set the **Method** to **Contrast** (only available option at this time).
- Set the **Z range** - this is the total range to search through for focus.

- Set the **Fine Focus Steps** - the number of steps to take during the second focus pass.
- Set the **Step Size** - the size of each step taken during the second focus pass.

To run the experiment:

1. Enter the appropriate file name and path into the **Data File** field. If you would prefer to navigate to the folder where you want to save the data file, click the **Save As** button to open a file browser window.
2. If desired, enter a short experiment description or title into the **Title** field. This description is saved to the experiment log file.
3. Click **Run** to start the experiment.

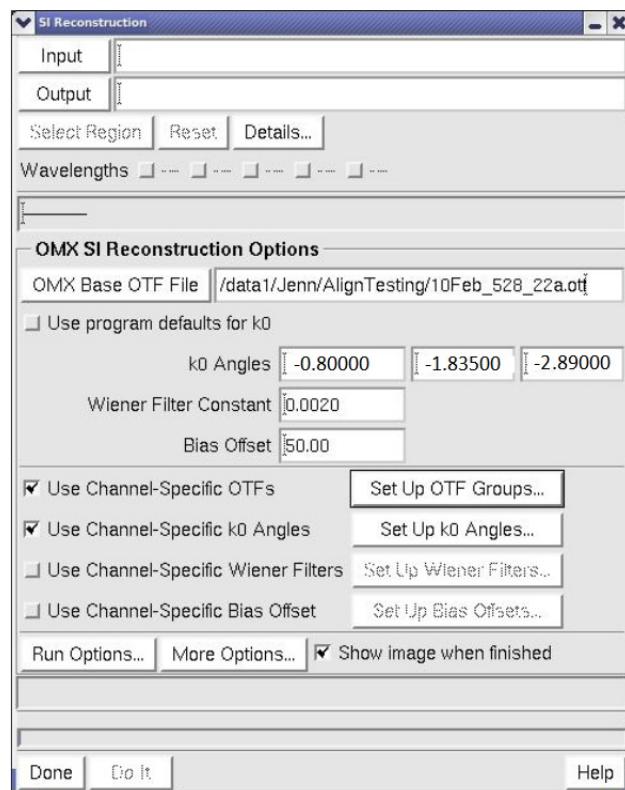
As the experiment runs, the experiment progress is displayed. When the experiment is complete, the stage controls are reactivated and, if you wish, you can set up and run another experiment.

To reconstruct the SI data:

This section describes how to use the OMX SI Reconstruction tool to reconstruct SI images.

1. In softWoRx, click **Process | OMX SI Reconstruction** to open the SI Reconstruction dialog box.

Figure 7. SI Reconstruction Window

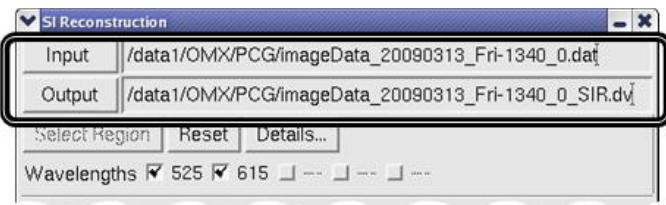


2. Specify an input file. Choose one of the following:

- Click the **Input** button and select a file from the displayed list. If necessary, use the **Directory** field and/or navigation icons in the Select File dialog box to navigate to the directory containing the desired file.
OR
- Drag-and-drop a file from the file browser window into the **Input** field.
OR
- Drag-and-drop a window identifier number from an open image window.
OR
- Type the path and file name directly into the **Input** field.



Note The **Output** field populates automatically once you specify an **Input** file name.



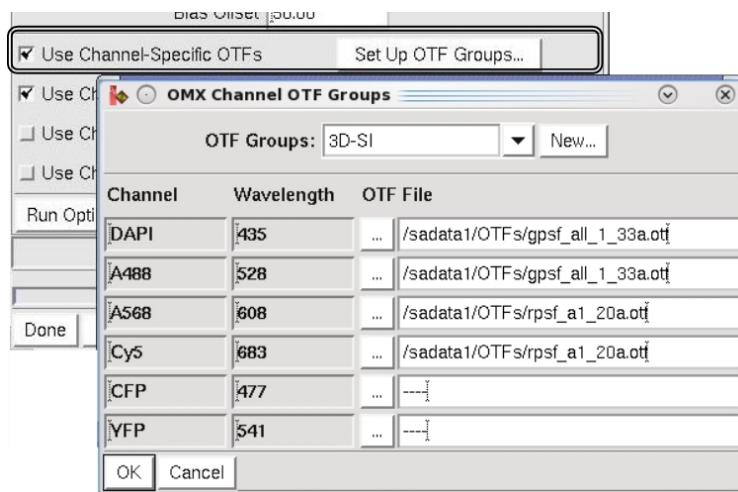
3. Specify an OTF Group to use for processing. For best results, it is recommended that you use channel-specific OTF files as follows:
 - a. In the bottom section of the SI Reconstruction Window, ensure that the **Use Channel-Specific OTFs** check box is selected.



Note After you select the input image, the software automatically selects the last used OTF for the input data type, and that group name is displayed on the button text. Either use that group or complete the following steps to modify the OTFs used.

- b. Click **Set Up OTF Groups** to open the OMX Channel OTF Groups dialog box.

Figure 8. Displaying OMX Channel-specific OTF Group Options



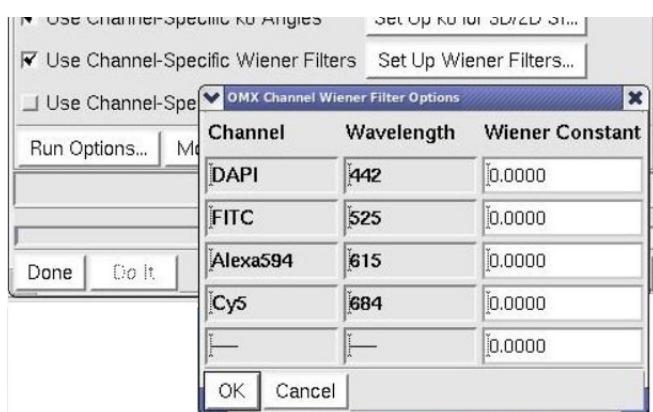
- c. In the **OTF Groups** field, use the drop-down button next to the field to select a previously used group name, or click the **New...** button next to the field to add a new group name.
- d. Select the correct OTF group.
- e. If the **File** field is not yet populated, click the ... button to select the OTF file to use for each channel.
- f. When you are satisfied with the OTF File settings, click **OK**.
4. Enable the **Use Channel- Specific k0 Angles** check box. To specify k0 angles for individual channels click **Set Up k0 3D/2D SI**. The OMX Channel k0 Options dialog box is displayed.

Figure 9. Displaying Channel-specific k0 Angle Options



5. Verify the appropriate values are present in the table, and click **OK**.
6. To use a single Wiener Filter for all channels, do not enable the **Use Channel-Specific Wiener Filters** check box. To specify Wiener Filters for individual channels, enable the check box and click **Set Up Wiener Filters**. The OMX Channel Wiener Filter Options dialog box is displayed.

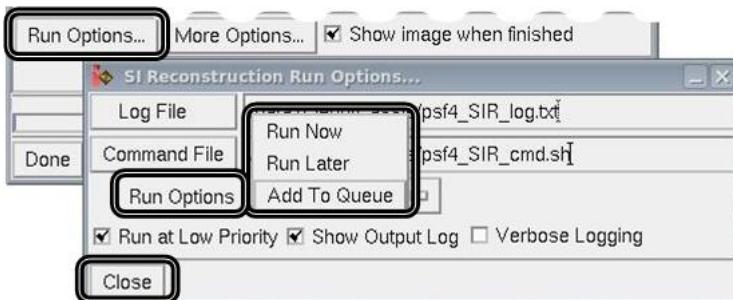
Figure 10. Specifying Camera-specific Wiener Filter Options



Note Channel-specific Wiener Filter values override the single value entered into the **Wiener Filter Constant** field. Typical values will range from 0.001 to 0.005. Higher ones may be considered for 2D SI reconstructions.

7. Select the **Wiener Constant** to use for each channel and click **OK**.
8. SI image reconstructions can be run immediately or they can be added to an image processing queue. The default setting is to “Run Now.” If you prefer, click **Run Options** to display the SI Reconstruction Run Options dialog box and select a different setting. Click **Close** to complete the process.

Figure 11. Specifying Run Options



9. If necessary, click **More Options** to open the More SI Options dialog box. For most samples, these options are not needed, but in some instances, they may assist in minimizing artifacts in the images. The More Options fields are described immediately after the following figure.

Figure 12. Specifying More Options

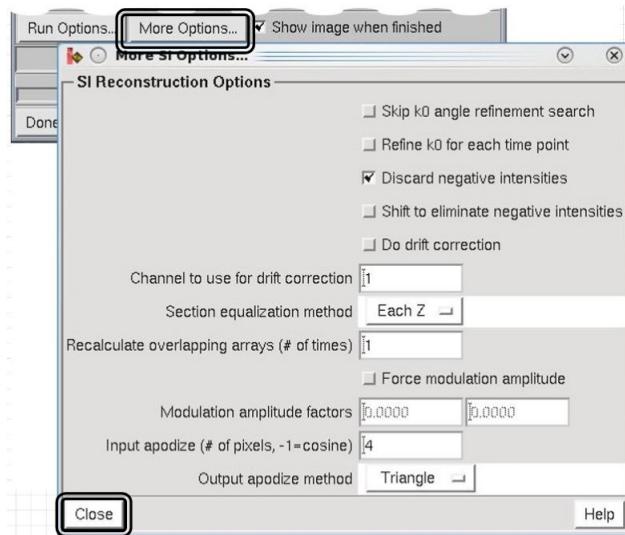


Table 1. More Options Fields

Field	Description
Skip k0 angle refinement search	Directs the reconstruction algorithm to assume the provided angles are correct and not to search for a better fit. If correct angles have been provided for all channels, activating this setting can improve reconstruction results for low signal data.
Refine k0 for each time point	Performs a k0 search for each time point. If disabled, the k0 search is performed only at the first time point for each channel and that value is used for all the remaining time points in that channel. This can increase the length of time required for reconstruction.
Discard negative intensities	At the end of reconstruction, sets all pixels with negative intensities to zero. This allows the default scaling in softWoRx to be more appropriate for the data sets.
Shift to eliminate negative intensities	At the end of reconstruction, increases all pixel values together so that the lowest intensity pixel is equal to zero.
Do drift correction	Prior to starting reconstruction, calculates and corrects for the sample drift between the first and second angles and the first and third angles. The drift values are calculated in one channel and the correction is applied to all channels. <i>This setting should be disabled for all systems configured with Blaze illumination.</i>

Field	Description
Channel to use for drift correction	Defines the channel used to calculate the drift. (1 = first channel in image, 2 = second channel, etc.). The selected channel should have high signal-to-noise. If no channel has high signal-to-noise, the drift correction should be disabled because an incorrectly calculated drift value adversely affects the reconstruction.
Section equalization method	Defines how varying intensities across the data will be corrected (primarily in sample bleaching). Select None when significant camera bias drift is encountered during imaging.
Recalculate overlapping arrays (# of times)	Defines the number of times overlapping arrays are calculated.
Modulation amplitude factors	Defines the first and second order modulation values. If these values are set to anything other than zero, the algorithm will not search for the modulation. Setting these values to "1.00, 1.00" can improve reconstruction results on samples that have high background fluorescence.
Input apodize (# of pixels, -1=cosine)	Defines the input apodize method (used in managing edge data).
Output apodize method	Defines the output apodize method (used in managing edge data).

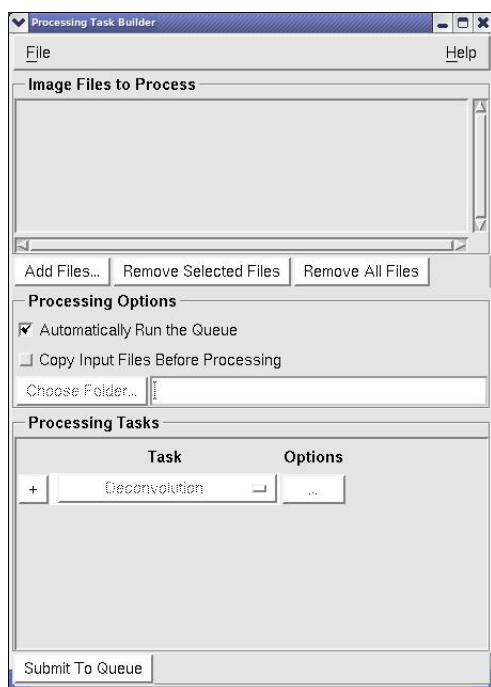
10. Set the **More Options** fields as needed and then click **Close**.
11. Click **Do It** in the main SI Reconstruction dialog box to start the reconstruction process.

OMX SI Reconstruction Using the Task Builder

This section describes how to use the Task Builder to reconstruct SI images.

1. Ensure the system has been turned on according to the startup procedure described in "System Startup" on Page 3.2 and that the softWoRx imaging software is running on the DeltaVision OMX SR Workstation.
2. In softWoRx, click **Process | Task Builder** to open the Processing Task Builder dialog box.

Figure 13. Processing Task Builder

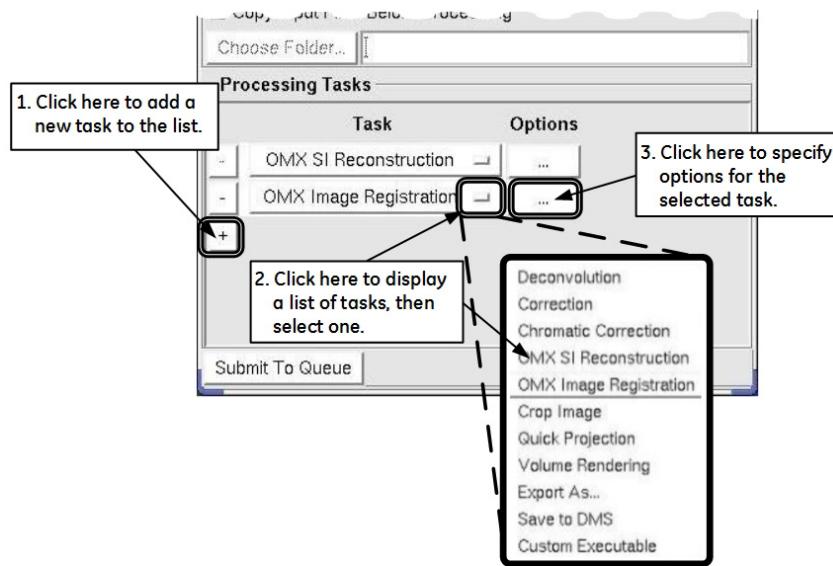


3. Select the image files to process. Choose one of the following:
 - o Click the **Add Files...** button and select a file from the displayed list. If necessary, use the **Directories** and **Filter** fields in the Select File dialog box to navigate to the directory containing the file.

OR

 - o Drag-and-drop a file from the file browser window into the **Image Files to Process** field.
4. Enable/disable the desired Processing Options:
 - o **Automatically Run the Queue.** Automatically start the specified processing tasks immediately after you click the **Submit to Queue** button. Disable this option to process the contents of the queue at a later time using the tools available in the softWoRx Queue Manager.
 - o **Copy Input Files Before Processing.** Specify a folder to copy the specified input files to before processing. The output files are saved to the same directory as the input files. This option is often used to move the input and output files to a cluster location in order to speed up processing and store data.
5. Select the tasks to run on the specified input file. Click the plus sign (+) to place a new task on the list, the minus sign (-) to delete a task, and **Options** to specify the parameters for that task.

Figure 14. Specifying Tasks for the Queue (in three steps)



Note Many processing tasks may be added to the task list. The task "OMX SI Reconstruction" is described in this document. Refer to the *softWoRx Imaging Workstation User's Manual* for descriptions of the other tasks.

-
6. Click **Submit to Queue**. Depending on whether or not you enabled the **Automatically Run the Queue** check box previously, the job will either start immediately after you click the **Submit to Queue** button, or when you choose to run it at a later time from the softWoRx Queue Manager.

For local office contact information, visit:

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