DeltaVision™ OMX

Customer Instructions

Image Alignment

DeltaVision™ OMX Image Alignment

- *Introduces the OMX Image Alignment Calibration*
- *Describes the procedure used to create and apply channel alignment parameters to all types of OMX data*

Important user information

WARNING
Using controls, making adjustments, or performing procedures other than those specified in the DeltaVision OMX Imaging System's documentation can result in hazardous exposure to high voltage, laser radiation, or moving parts. Exposure to these hazards can cause severe personal injury and/or cause damage to the equipment. Do not operate the DeltaVision OMX Imaging System in any way other than described in the user documentation.

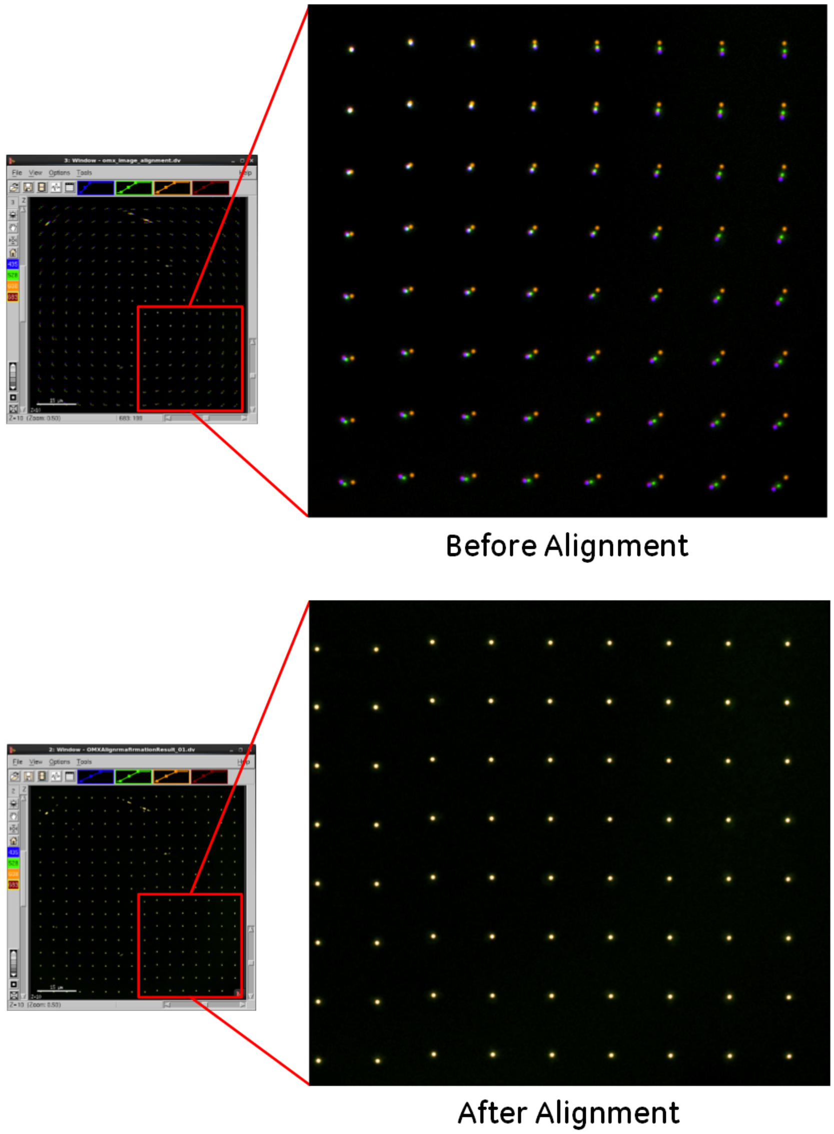
IMPORTANT!
Prior to reading this document, you must read and understand the safety and operating information described in the *DeltaVision OMX Operating Instructions* and the *DeltaVision OMX Getting Started Guide*.

Introduction

Camera filters and lenses are manufactured to specifications that define a range of tolerance. The smaller the tolerance range, the more expensive the glass. No matter how expensive the glass, though, or how small the tolerance range, microscopic imperfections exist in filters and lenses that may cause distortions in your final image. Additionally, a multi-camera system like the OMX requires a method to correct for slight differences in X/Y position and focus between images acquired using different cameras. The OMX Image Alignment procedure uses images of a calibration slide to map each wavelength for the displacement caused by these imperfections and then uses the resulting maps to create a mathematical model to compensate for these effects in your images.

The following images show the alignment grid on the Image Alignment Slide before and after the alignment calibration has been completed.

Figure 1. Before and After OMX Image Alignment Calibration (Same Colors in All Images)



The DeltaVision OMX uses the Image Alignment Slide (also referred to in this topic as the "alignment slide") to complete the OMX Image Alignment calibration procedure. This slide incorporates a transmitted light target consisting of four arrows that point towards each other and the center of the slide/coverslip. A 20 x 20 grid of 100 nm holes with 5 µm spacing is located in the center of the slide/coverslip, 1mm from the tip of each arrow.

Figure 2. Image Alignment Target

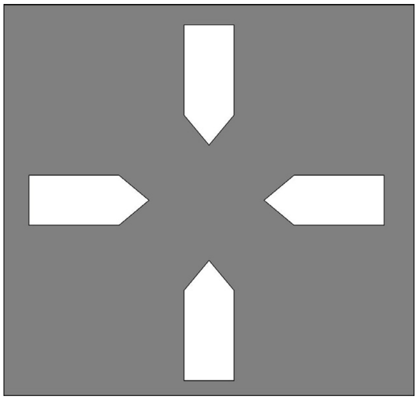
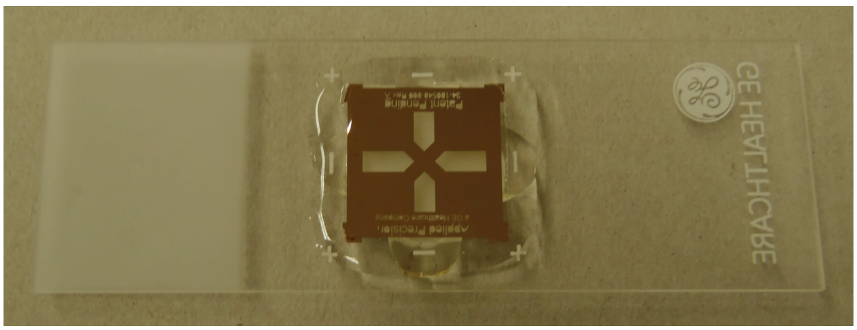


Figure 3. Image Alignment Slide, Target Faces Toward Objective



Alignment Methods

The DeltaVision OMX has always included a method for image alignment but the specific alignment algorithms have evolved over time. softWoRx 7.0 incorporates the newest and most accurate image alignment method. Legacy methods are still available in the software for reprocessing old data and can be found by selecting **Process | Legacy**. The current and legacy methods are briefly described in this section.

Current OMX Image Alignment (Local Triangulation) Method

The current alignment method uses a two-step process to correct both global misalignment and local non-linear misalignment. The calibration data is used to create a list of matched fiducials between the reference channel and the channel to be aligned. The matched fiducials are first used to apply a global alignment, correcting misalignment across the entire field of view. Localized warp alignment follows, using a triangulation of the local fiducials to apply fine alignment.

Legacy OMX Image Registration (X and Y Best Fit) Method

This method iteratively calculates a best fit polynomial along both axes. It corrects for image shift and magnification changes but does not correct for non-linear local misalignments. For instructions on how to use this method, see Appendix A later in this document.

Legacy OMX Alignment Parameters (Shift and Rotate) Method

This method applies vertical and horizontal shifts as well as rotation about the center of the image to align wavelengths. It does not account for magnification or non-linear misalignments between wavelengths.

Performing the Alignment

Several steps are required to complete this process.

- Mount the Image Alignment Slide
- Locate the grid of holes
- Acquire a Z stack of the image alignment grid
- Create an OMX Image Alignment calibration file
- Finalize axial alignment using the Chromatic Correction tool and a TetraSpeck™ bead slide[1]

Each of these steps is described in detail in the sections that follow.

Prerequisite

- softWoRx version 7.0 or higher must be installed on the OMX workstation

Tools

- Image Alignment Slide
- TetraSpeck™ bead slide

Procedure

To mount the alignment slide:

1. Open the door to access the microscope. The laser interlock will keep the safety shutter closed while the door is open.

WARNING! Due to the potential for exposure to hazardous radiation, do NOT defeat the laser interlock.

2. If necessary, remove any sample in the slide holder and clean the objective.
3. Apply the immersion oil best matched to the beads on the coverslip in your reference channel to the objective. If you are not sure of which reference channel to use, start with 568.
4. Place the alignment slide, target facing down, in the slide holder.
5. Pivot the transmitted light tower forward so that it is positioned over the slide. Ensure that the transmitted light aperture is open. Remove the green filter and diffuser

1. TetraSpeck is a trademark of Thermo Fisher Scientific.

located in the filter sliders on the left side of the transmitted light arm, below the LED, from the transmitted light path.

6. Using the joystick, a stored Z-touchdown position, or the Nano Positioning tools located in the Acquire SR software, position the stage so that the immersion oil on the objective is touching the slide and the center of the objective is positioned within one of the four arrows on the alignment slide.
7. Close the door to the Microscope Enclosure.

To locate the grid of holes:

1. Click **File | Settings** and then, in the **Objective** field, select the objective lens for which the image alignment is being calibrated.
2. Activate a single channel by clicking the appropriate **Channel** button. An image window will open for the activated channel.

Important To calibrate all channels together, set **Image Mode** to “Sequential.”

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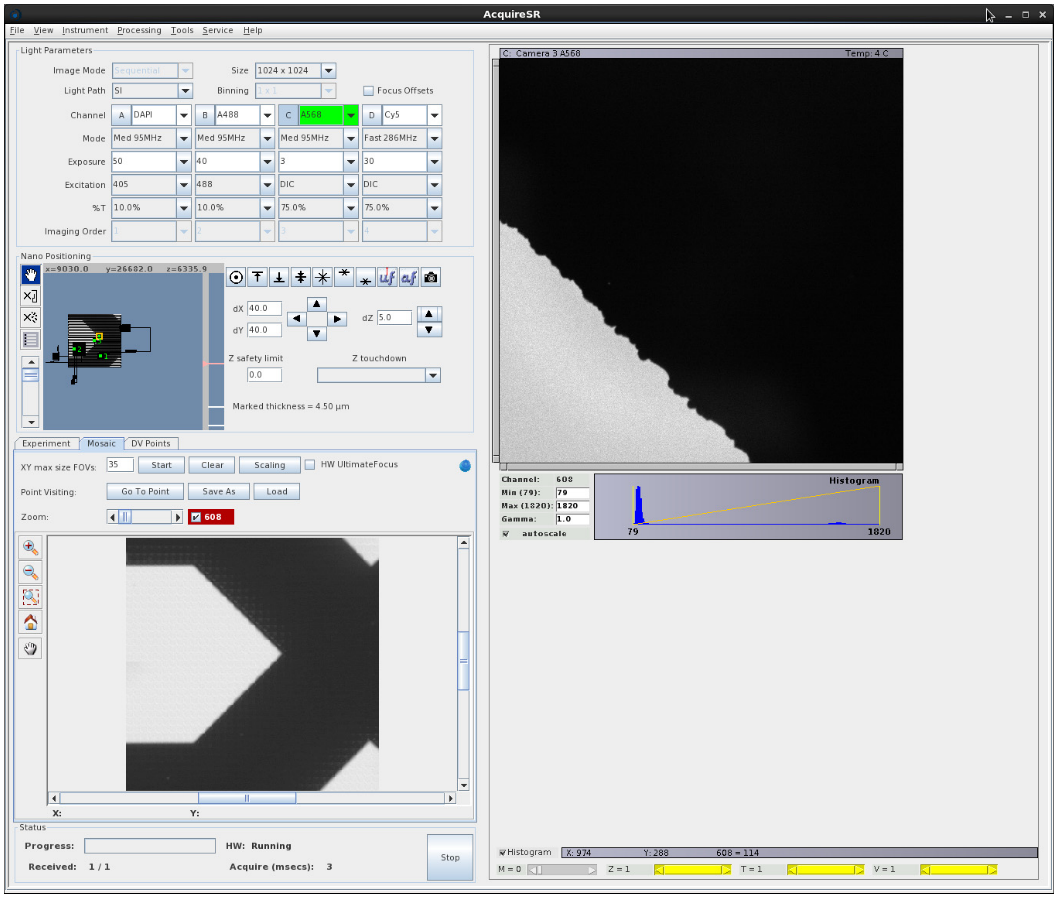
3. In the **Light Path** field, select the “Conv” setting.
4. In the Light Parameters section, ensure that **Focus Offsets** is not selected.
5. For the activated channel, select an emission filter from the drop-down list.
6. Select “DIC” for the **Excitation** setting.
7. Select a **%T** setting and exposure time to allow sufficient light to the camera.
8. Find the edge of one of the arrows using one of the following methods:
   - Use a previously stored position from a points list

     OR
   - Move the stage to a previously recorded stage position using the **Instrument | Move Stage** command

     OR
   - Use the Nano Positioning tools and/or the Mosaic utility.

Figure 4. Find the Edge of an Arrow



9. Once you find the edge of an arrow, use the Nano Positioning tools to follow the edge until you find the point (tip) of the arrow.

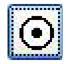
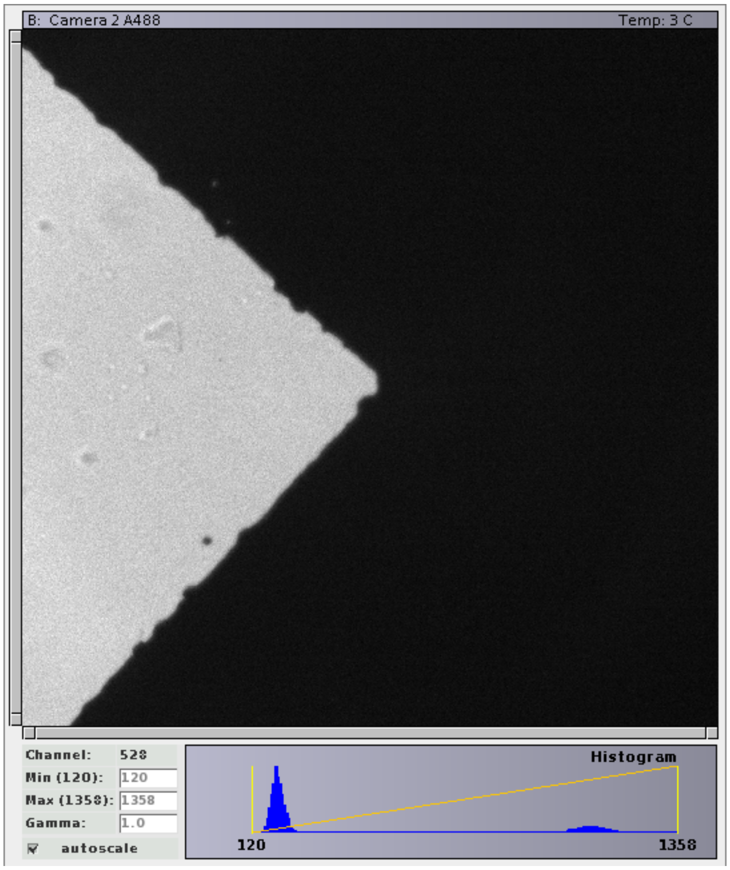
10. Use the Center Point button  to center the tip of the arrow in the viewing window.

Figure 5. Center the Tip of the Arrow



Important If you have not previously done so, record the X/Y/Z coordinates for the tip of the arrow and the orientation of the alignment slide on the stage to streamline the Image Alignment procedure in the future. Focusing on the grid of holes at the center of the slide is difficult so it is not recommended to save that X/Y/Z location.

11. Move 1 mm (1000 µm) in the direction the arrow is pointing. If you move correctly, you will see the grid of holes. For example, in the previous figure, you would move 1000 µm to the right in the X direction.

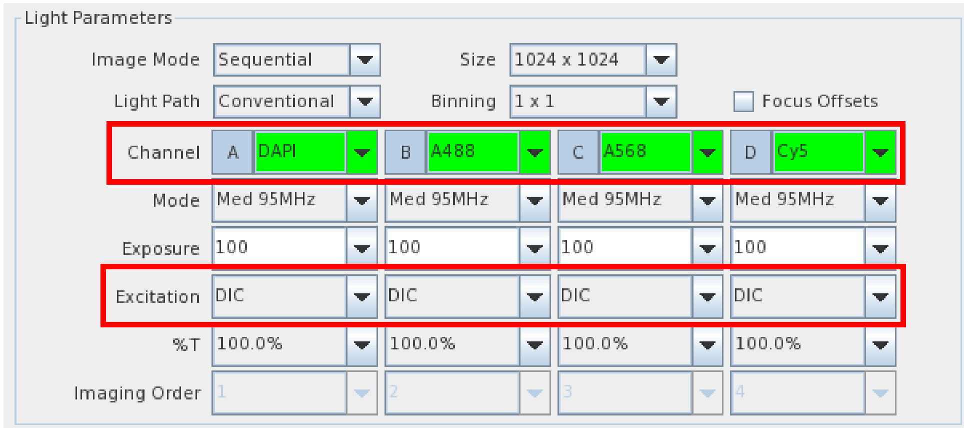
Note The grid of holes with 5 µm spacing is surrounded by a larger grid of holes with 10 µm spacing. If you see both grids within the field of view, reposition using the Centering Tool or the X/Y arrows until only the smaller grid (5 µm spacing) is visible.

12. Focus on the holes.

To acquire a Z stack of the image alignment grid:

1. Set the field of view **Size** to 1024 x 1024. (For EMCCD cameras, use 512 x 512.)
2. Activate all the cameras and set each camera's **Excitation** to "DIC."

Figure 6. Activate All Cameras and Set Excitation to "DIC"



3. Refine **Exposure** for each channel to target a maximum intensity of about 3000 counts.

Tip The Image Alignment calibration works best if all channels have a similar dynamic range although this can be hard to achieve on systems where two or more channels share a camera. In this case, ensure that no channel is saturated and that all channels have at least 1000 counts over background.

4. On the Experiment tab:
   a. Set the experiment **Type** to "Conv."
   b. Set the **Optical section spacing** to "0.125."
   c. Set the **Sample Thickness** to "3" microns.

Tip Ensure that the focal plane for all channels will be captured within the specified thickness.

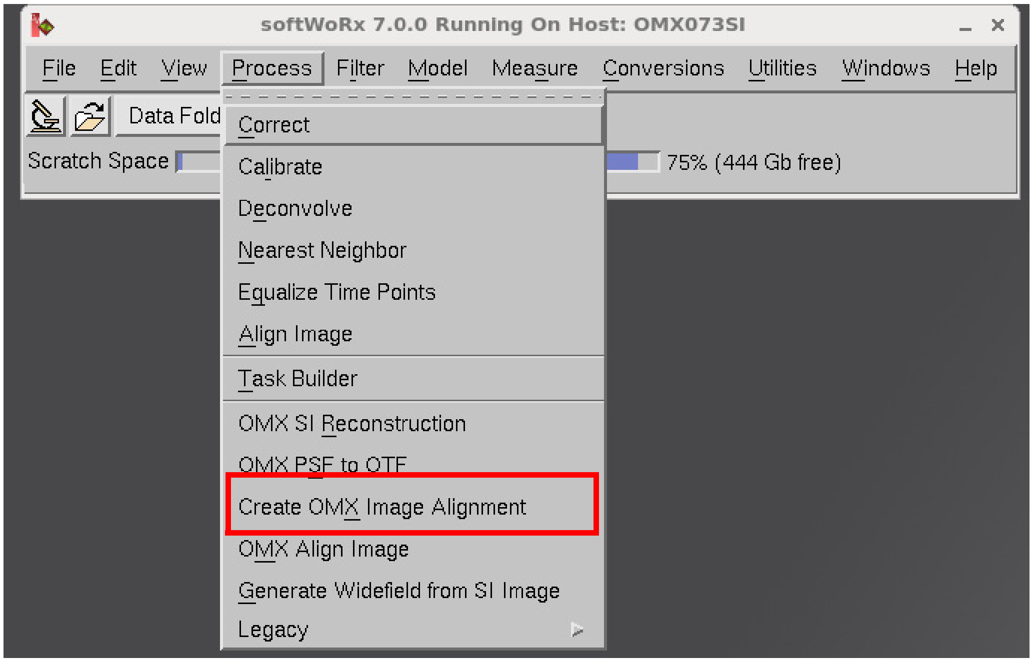
   d. Enter a filename in the **Data File** field.

5. Click **Run** to start the experiment.

To create an OMX Image Alignment calibration file:

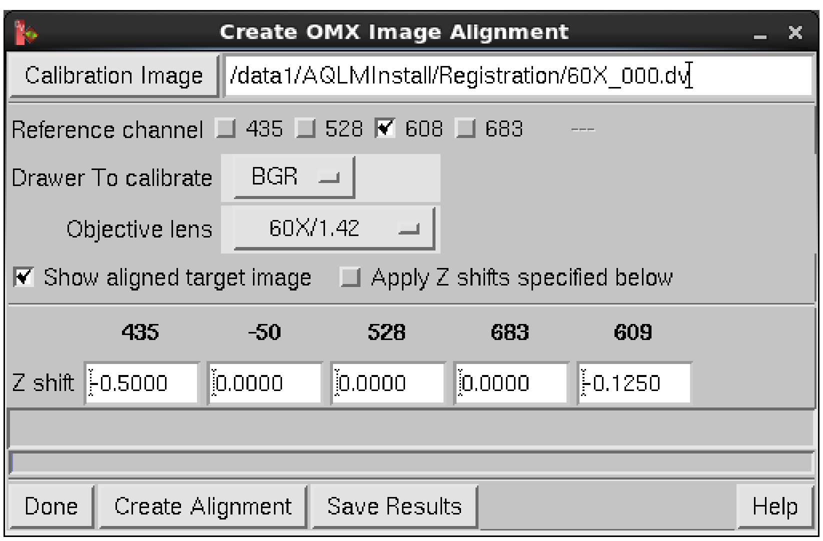
1. From the softWoRx main menu, click **Process | Create OMX Image Alignment**.

Figure 7. Process Menu



The OMX Image Alignment parameters are displayed.

Figure 8. Create OMX Image Alignment Parameters



2. Enter the name or the window ID number of the .dv experiment file created in the previous section into the **Calibration Image** field.
3. Select the most commonly used imaging channel as the **Reference Channel**. If you are not certain, use the red (608) emission channel.
4. Specify the correct **Drawer to Calibrate**. This setting must match the drawer used to acquire the calibration data.

5. Verify that the **Objective Lens** matches what was used to acquire the calibration data.
6. Ensure the **Show aligned target image** check box is selected and that the **Apply Z shifts specified below** check box is clear.
7. Click **Create Alignment**. The tool will use the input image to create an alignment calibration then it will align the input image.
8. Examine the original image of the calibration slide, the alignment calibration log file, and the aligned image to ensure that satisfactory results were achieved.
   - ***Original image:*** After creating the alignment calibration, circles showing the detected fiducials will be displayed in the original image of the calibration slide for all channels except the reference channel. Examine the number and location of the fiducials in each channel. Have fiducials been detected across the entire FOV for all channels?
   - ***Alignment calibration log file:*** In the log file that opens during alignment, scroll to the top of the window and examine the number of peaks that were found during alignment. The more peaks identified, the better the alignment. For a 1024 x 1024 FOV, > 250 is very good, 200-250 is good. If fewer than 200 peaks were found, this may suggest that dynamic range for one or more channels was too low.
   - ***Aligned image:*** Display all channels (**View | Blended Colors**) for images with four channels) and closely examine the image, especially at the corners. Do the holes in each channel seem co-localized?

   If you are not satisfied with the results, verify parameters in the **Create OMX Image Alignment** window or change the **Reference Channel** and re-run the alignment calculation as needed. If that does not work or if there were very few peaks detected in one or two channels, re-image the grid slide with increased %T and exposure time to improve dynamic range.
9. When you are satisfied with the results, click **Save Results**.

Tip Calibration results are saved as a text file in the /home/<username>/softWoRxconfig directory. When Align Image is used during an imaging session, this information is also saved to the ParameterFiles folder within the current data folder.

To finalize axial alignment using the Chromatic Correction tool:

Important If you have previously completed the Z alignment for your DeltaVision OMX, you should not have to repeat this portion of the procedure unless you have recently had a camera replaced or realigned by GE Service engineers. Stored Z shifts that work for most samples, but not a particular sample, suggests an issue with the sample itself. Verify that the sample is mounted without any tilt (often caused by tape, labels, or the coverslip located on only one end of the slide), that the sample is mounted directly onto the coverslip, and that the oil matching is appropriately correcting the spherical aberration.

1. When you are satisfied with the XY Registration Calibration results, turn on the Laser Control Module and switch back to the AcquireSR software.
2. Using an immersion oil with a refractive index (RI) that is matched to the reference channel, mount a TetraSpeck bead slide on the stage.

3. In the **Light Path** field, select "SI".
4. Focus on the sample and find a field of view containing a number of beads.
5. Activate all channels and refine **%T** and **Exposure** to target about 5000 counts for each wavelength. If you cannot achieve greater than 2000 counts in any channel, switch the **Light Path** setting to "Conv".
6. On the Experiment tab:
   a. Set the experiment **Type** to "SI".
   b. Set the **Optical section spacing** to "0.125".
   c. Set the **Sample Thickness** to "3" microns.
   d. Enter a file name in the **Data File** field.
7. Click **Run**.
8. In softWoRx, reconstruct the TetraSpeck bead image.

Tip Using a 3D SIM stack allows for increased accuracy when sufficient intensity can be collected. If a widefield conventional stack was collected instead, deconvolve the raw data.

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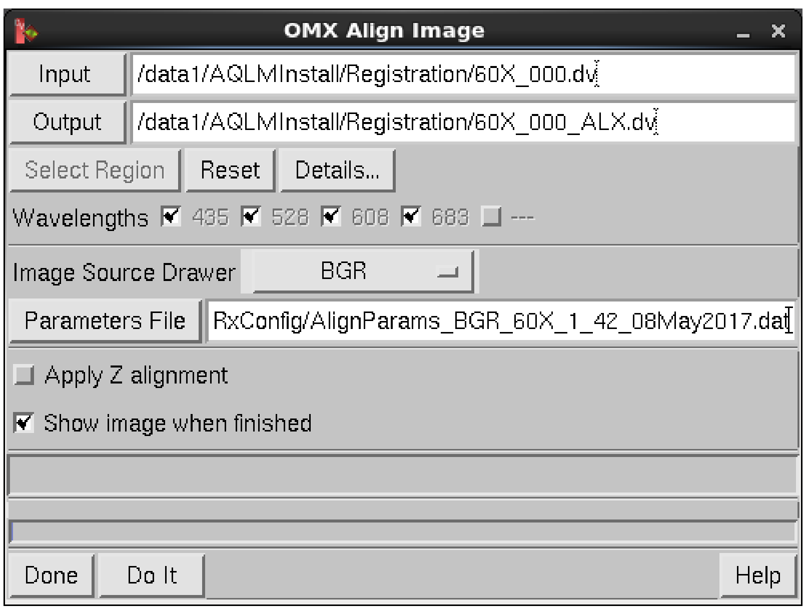
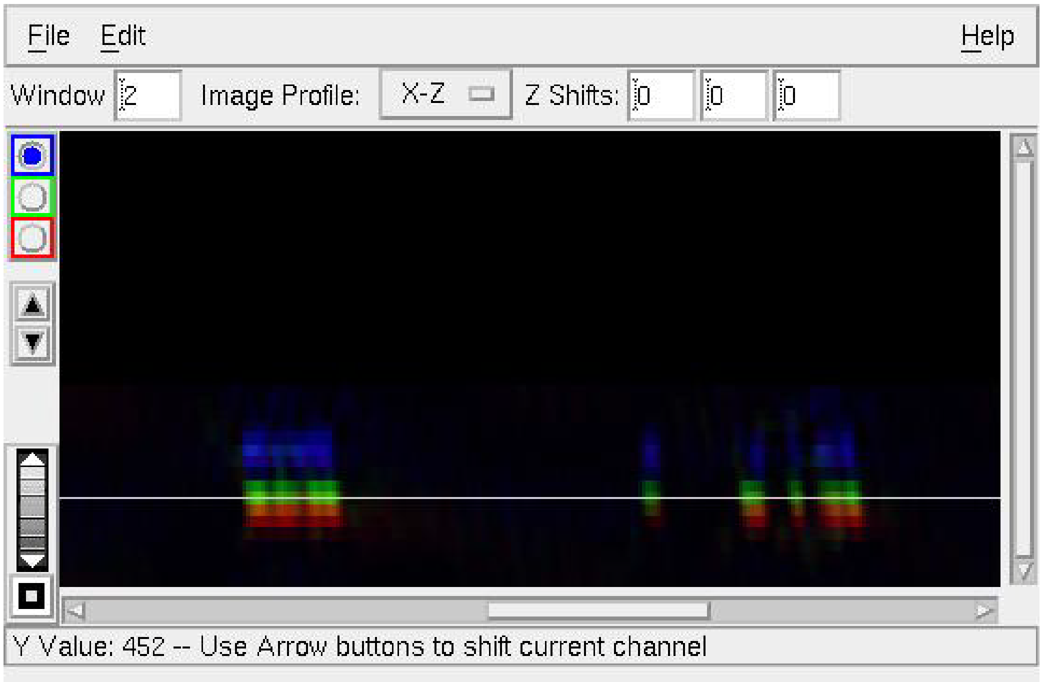
9. Select **Process | OMX Align Image**. Clear the **Apply Z alignment** check box and select the **Show image when finished** check box. Click **Do It**.

Figure 9. OMX Align Image



10. Select **Measure | Chromatic Correction** and select the reconstructed and aligned TetraSpeck bead image as the input.

Figure 10. Select the TetraSpeck Bead Image as the Input

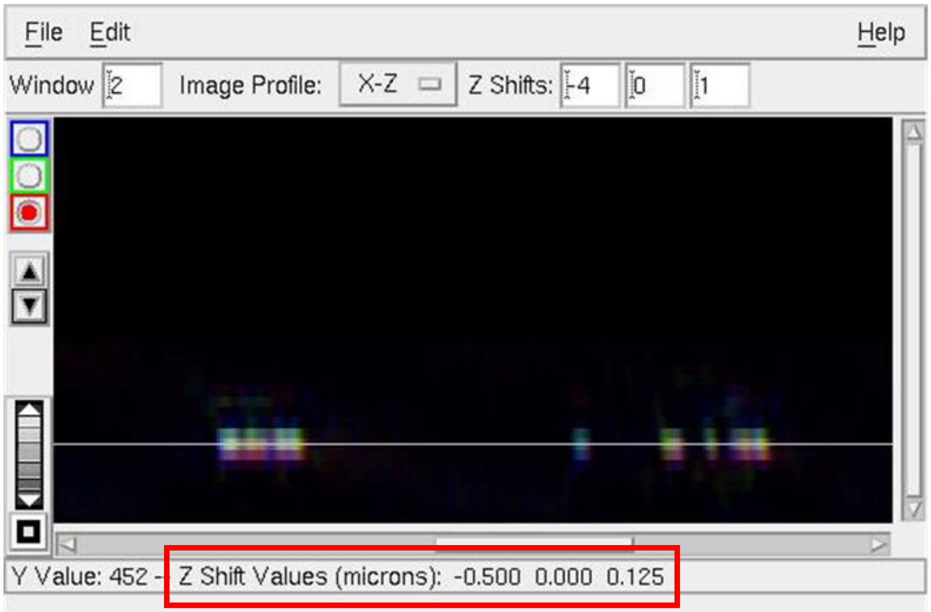


Note The Chromatic Correction tool can only display three channels. If you are working with more than three channels, you must use the following steps to correct all channels:

1. Use the original reconstructed TetraSpeck bead image to correct the first three channels in the image file, using the same reference channel from the previous steps in this document.
2. Open the reconstructed TetraSpeck bead image file and select **File | Save**.
3. Select only the reference channel and the fourth/last channel and save the image.
4. Use this image in the Chromatic Correction tool to find the Z offsets for the remaining channel.

11. Using the arrow buttons and channel selectors along the left side of the Chromatic Correction tool, shift the channels so that they line up in Z with the reference channel. Use the same reference channel here as was selected for the XY alignment parameters.

Figure 11. Shift the Channels to Line Up in Z with the Reference Channel (green in this example)

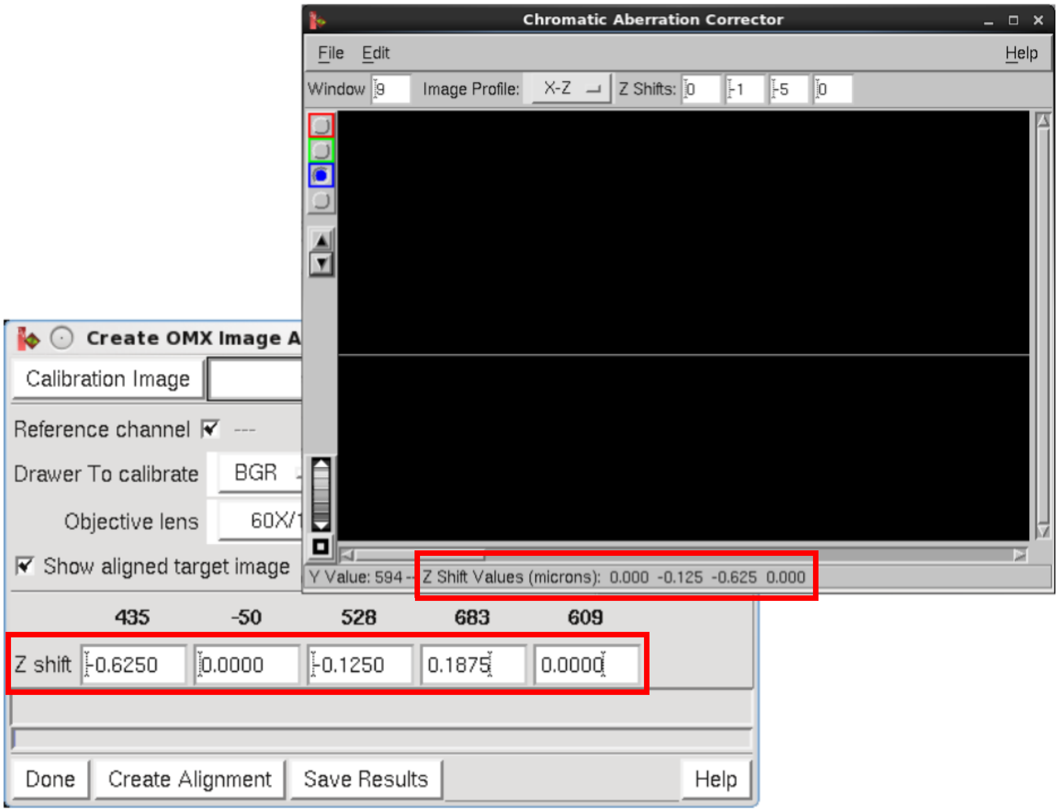


12. Enter the Z Shift Values displayed at the bottom of the Chromatic Correction tool into the **Z Shift** fields for each channel in the Create OMX Image Alignment window. You can also calculate the total Z shift by multiplying the total number of Z sections to shift by the Z section thickness, 0.125 µm.

Note The Chromatic Correction tool only allows for whole Z section shifts; however, the Create OMX Image Alignment tool allows for sub-section Z shifts. Simply enter the required distance to shift in microns and the resulting pixel values will be interpolated as necessary to provide the sub-section shifts.

In the figure that follows, the shift for the 683 nm channel was more than one section (0.125) but less than two sections (0.250) so a Z shift equal to 1.5 sections (1.5 x 0.125 = 0.1875) was used.

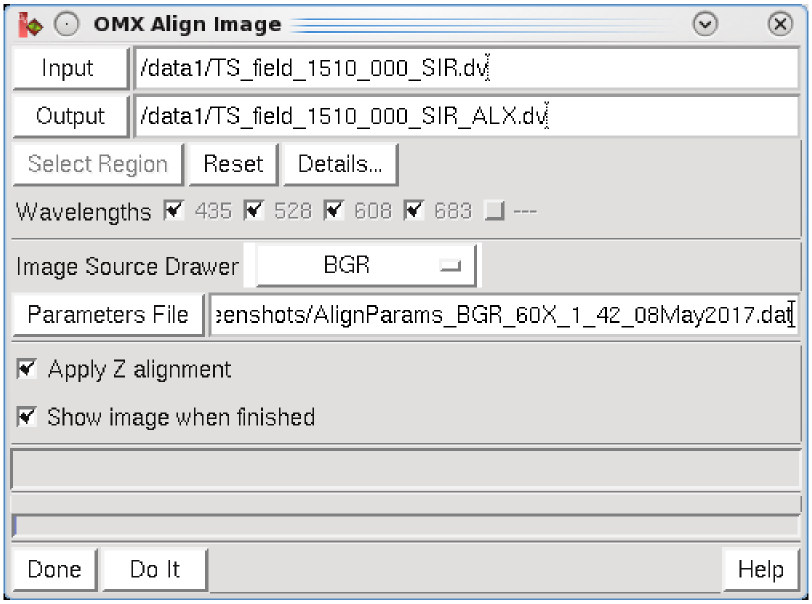
Figure 12. Enter Z Shift Values From the Chromatic Correction tool into the Create OMX Image Alignment Window



13. Select **Apply Z shifts specified below** and click **Save Results** to save the Z shifts.
14. Select **Process | Align Image** and select the reconstructed, un-aligned TetraSpeck bead image as the input.
15. Verify that the correct **Image Source Drawer** is populated.

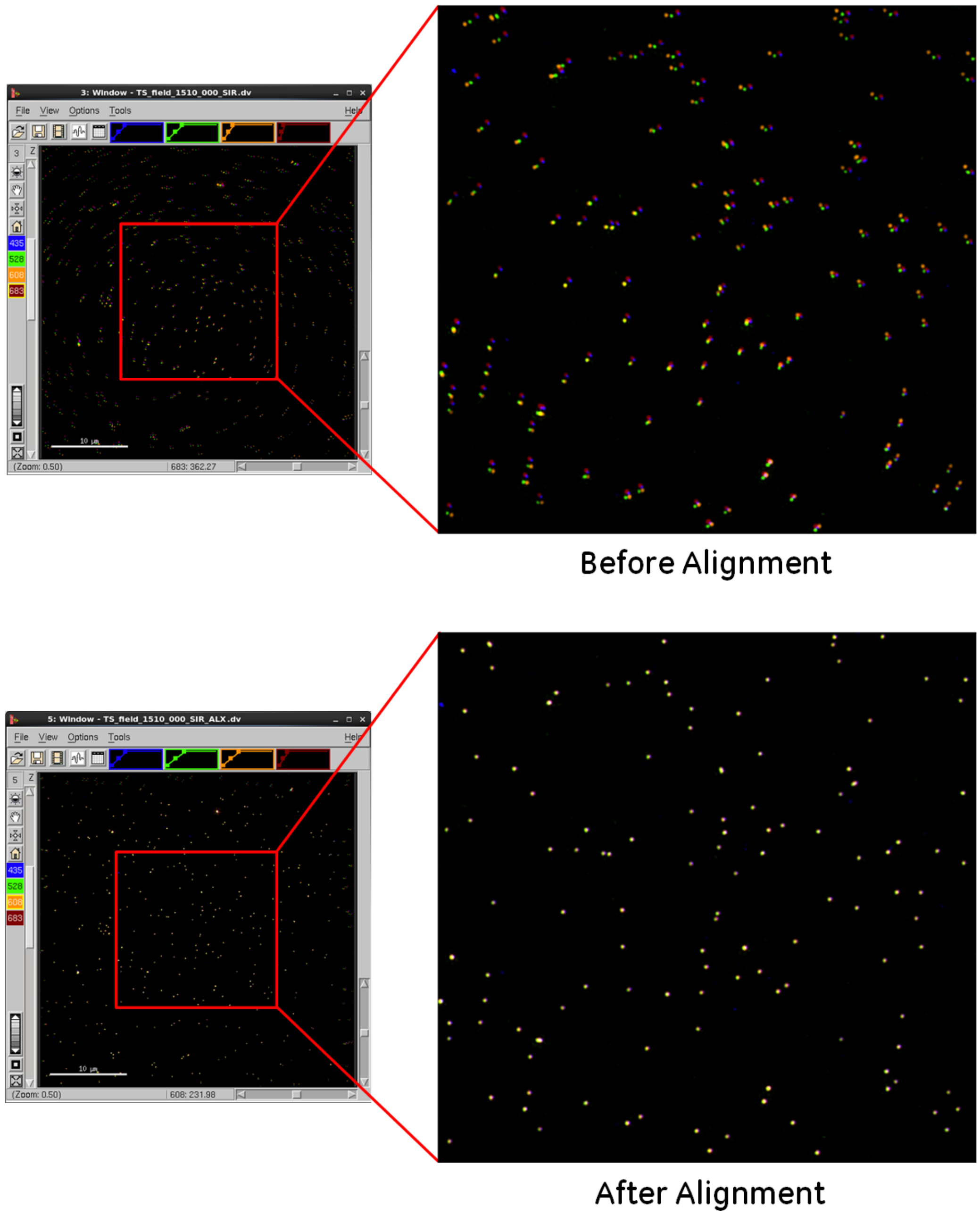
16. Select **Apply Z alignment** and **Show image when finished**.

Figure 13. Select Apply Z Alignment

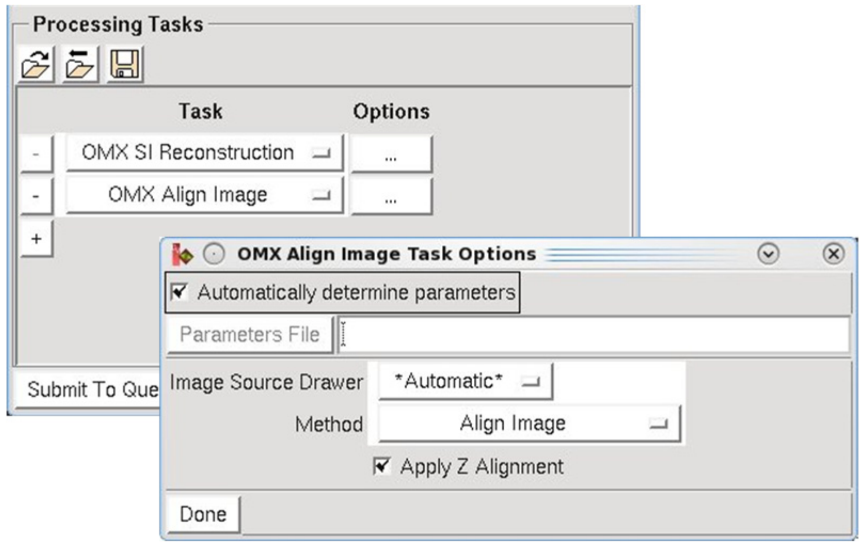


17. Click **Do It**. After the alignment is complete, check that the center of the TetraSpeck beads are aligned laterally and axially in all channels. The aligned image should appear similar to the "After Alignment" image shown in the lower-right corner of the following figure.

Figure 14. Before and After Alignment (Magnified Content in Images on Right)



Note Image alignment can also be applied from the softWoRx Task Builder. In the Task Builder, select "OMX Align Image." In the Options dialog box, select the **Automatically determine parameters** check box, the "Align Image" **Method**, and the **Apply Z Alignment** check box.

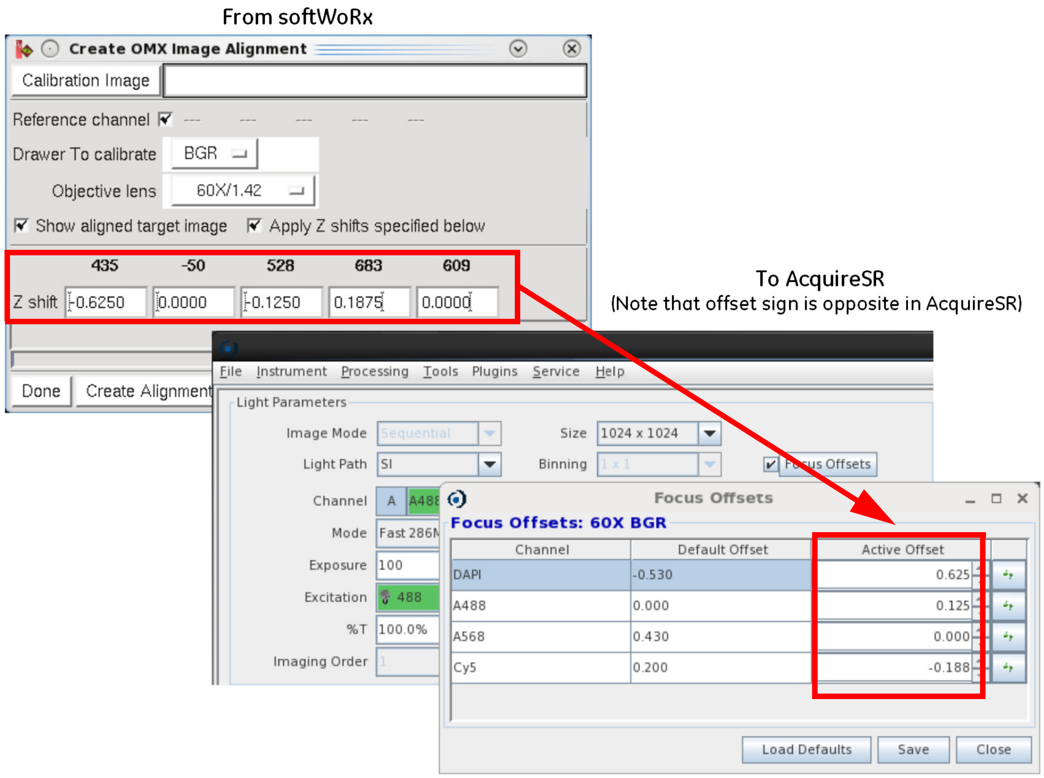


Important The image registration process involves pixel interpolation. Due to this interpolation, ensure reconstruction and deconvolution tasks are performed prior to image registration tasks.

18. Once you are satisfied with the Z shifts, enter them into the AcquireSR software for use during acquisition. In the Light Parameters section, select the **Focus Offsets** check

box, then click the **Focus Offsets** button. Enter the Z shift values for each channel into the Active Offset fields.

Note The sign of the offset should be opposite the sign of the value determined in the Chromatic Correction tool, as shown in the following figure.

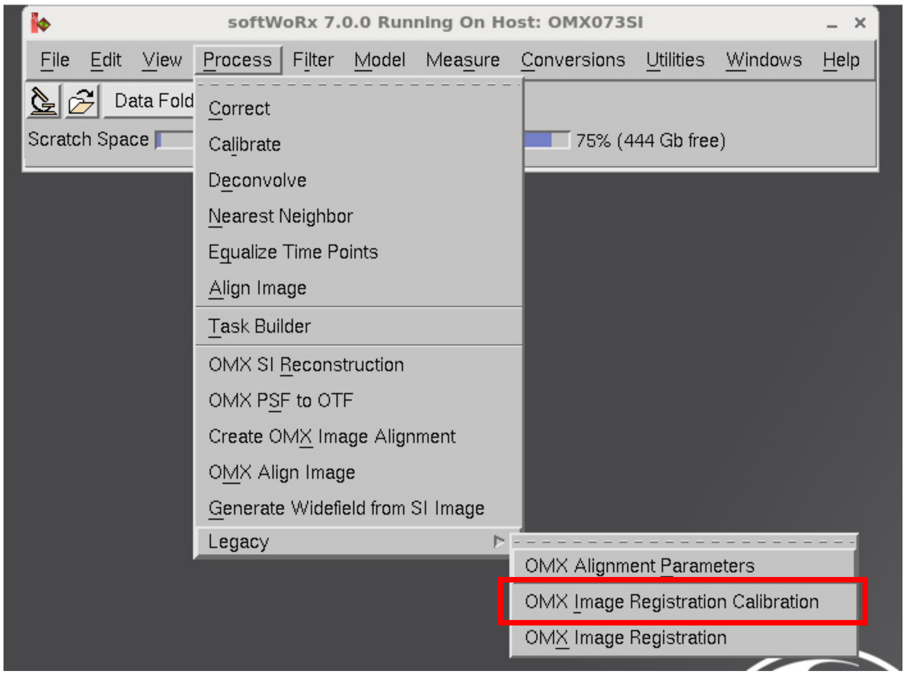


Appendix A: X and Y Best Fit Image Alignment (Legacy Method)

To create an OMX Image Alignment file using the X and Y Best Fit method:

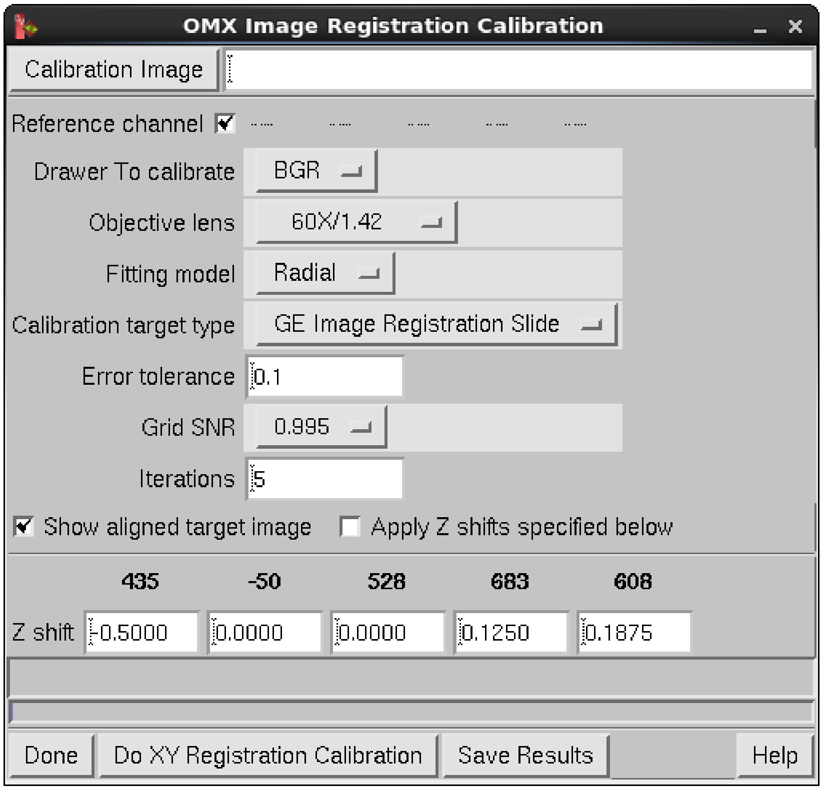
1. From the softWoRx main menu, click **Process | Legacy | OMX Image Registration Calibration**.

Figure 15. Legacy Menu



The OMX Image Registration Calibration parameters are displayed.

Figure 16. OMX Image Registration Parameters



2. Enter the name or the window ID number of the .dv experiment file into the **Calibration Image** field.
3. Select the most commonly used imaging channel as the **Reference Channel**.

Tip If one channel seems to be more challenging to align to the others, this may be due to slight differences in the optical path for that channel. Consider selecting that challenging channel as the Reference Channel to see if results improve.

4. Specify the correct **Drawer to Calibrate**. This setting must match the drawer used to acquire the calibration data.
5. Select “Radial” as the **Fitting Model**.
6. Specify “GE Registration Target” as the **Calibration Target Type**.

Note If you are using a TetraSpeck bead slide instead of GE’s Image Registration Alignment Slide, select “Other” as the **Calibration Target Type**.

7. Specify an **Error Tolerance** (in pixels). For most system configurations, a setting of 0.1 works well. This setting is the average error of the channel matching across the image and the iterations will stop once this tolerance is reached.
8. Specify a **Grid SNR** value. GRID SNR is an estimate of the contrast between the holes and the background in the alignment image. Typical values to start are 0.990 or 0.985. If the registration process takes more than five minutes to run, try a lower value.

9. Specify the number of **Iterations** to perform (typically 5). Once the algorithm search converges, or all of the iterations are complete, the system will save the "best fit" iteration results. If all iterations are used and the resulting alignment is not adequate, add more iterations, try a different **reference channel**, or try a lower **Grid SNR** value.
10. Ensure the **Show aligned target image** check box is selected and the **Apply Z shifts specified below** check box is clear. This instructs softWoRx to display the resulting aligned image after the calibration algorithm runs.
11. Ensure the **Apply Z shifts specified below** check box is clear.
12. Click **Do XY Registration Calibration** to run the calibration algorithm.
13. View the aligned image to ensure that satisfactory results were achieved. If you are not satisfied with the results, re-run the alignment calculation as needed. You can adjust the **Reference Channel**, **Fitting Model**, **Iterations**, and **Grid SNR** fields. You can also acquire another Z stack from the grid slide for input after first increasing the signal-to-noise in the images.
14. When you are satisfied with the results, click **Save Results**.

Tip Increase the signal-to-noise ratio in the image by either increasing the exposure times for each channel or by increasing the %T for the DIC light. Ensure that the signal from the holes/dots does not saturate the camera.

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