Adding an Objective

Before you begin:

Purpose: This procedure will demonstrate the steps required to add an objective lens to the DeltaVision system. It will show how to measure pixel size and add new lens information to the Lens dropdown in the Resolve3D window, as well as how to collect a Point Spread Function (PSF) and convert it to an Optical Transfer Function (OTF) for use in deconvolution. Lastly, this procedure will show how to associate an experimental OTF with a new objective.

1. Calibrating the Pixel Size

1.1. Make the adjustments listed in the table below:

Pixel Size Calibration	
Excitation Filter	TRITC
Emission Filter	FITC
Eyepiece Filter	FITC
% Transmission	1%
Beam Selector	Eye
Auxillary Magnification	1
Image Size	1024x1024
Stage	Circular Insert

1.2. Mount the grid slide:

1.2.1. Select oil with Refractive Index of 1.514 or 1.516 (for systems at 25ºC) and place one drop carefully onto the lens of the objective. If the objective is an air or water objective, skip this step.

NOTE: If your system is not at 25ºC, use the oil calculator to adjust oil refractive index accordingly.

1.2.2. Place the grid slide mirror-down over the keyhole cutout of the stage insert and use the course focus knob to bring the objective up to the slide.

1.2.3. Open the excitation shutter from the keypad. Through the oculars, focus on the grid etched into the slide.

1.3. Switch the Beam Selector to the camera and acquire an image. Make sure the Data Collection window is fit to screen  .

1.4. Acquire images as you carefully move the grid slide by hand to align the grid horizontally and vertically within the image window.

NOTE: There are multiple grids on the mirror slide. Make sure that the field of view contains one continuous grid.

1.5. Adjust the exposure time and %T to achieve a maximum intensity of about 2000.

1.6. Use controls in the Resolve3D window and adjust Z position to find the best focal plane.

1.7. In the Data Collection window, select **Tools > Measure Distances**.

1.8. Set the parameters in the Measure Distances window as shown below in Figure 1B:

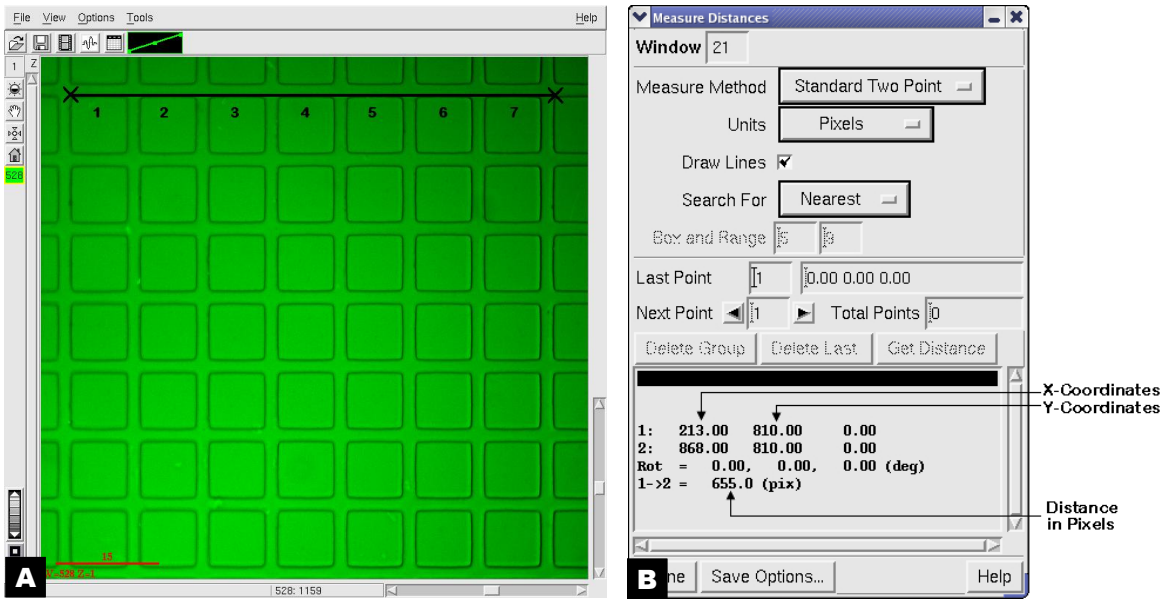


Figure 1. An acquired image of the Grid slide and the Measure Distances window. A) Data collection window with the measure distances line for the 7 grid elements shown. B) Measure Distances window with correct adjustments (highlighted with black boxes).

1.9. Draw a horizontal line to measure distance by clicking once on the left corner of the top left square and then clicking once on the left corner of the top right square as shown in Figure 1A.

1.10. Check that the line is as close to horizontal as possible by examining the difference in the Y coordinates between the two points. If the Y coordinates differ by more than 4 pixels, return to step 1.4 to re-align the grid slide.

1.11. Count and record the *Number Of Grid Elements* (boxes) measured.

1.12. From the Measure Distances window, record the measured distance in Pixels.

1.13. Repeat this process for the same *Number Of Grid Elements* from a row at the middle and at the bottom of the image.

1.14. Repeat steps 1.9–1.13 for three lines in the vertical direction. Make sure the vertical measurements span the same *Number Of Grid Elements* that the horizontal measurements spanned.

1.15. Average the 6 pixel measurements recorded (3 horizontal and 3 vertical) to get the *Avg Measured Pixels* value.

1.16. Calculate pixel size using the formula below:

$$Pixel\ Size\left(\frac{\mu m}{pixel}\right) = \frac{\left(9.995\frac{\mu m}{box}\right) \times \left(Number\ Of\ Grid\ Elements\right)}{Avg\ Measured\ Pixels}$$

1.17. Repeat procedure for each camera included with your system

2. Adding Lens Information to the Resolve3D window

2.1. Selecting the Lens ID from Lens Database:

2.1.1. In the main *softWoRx* window, select **Utilities > Revise Lens Database.**

2.1.2. Find the appropriate lens by matching the manufacturer, magnification, numerical aperture, and lens details to your new objective. Record the lens ID number.

NOTE: If the new lens is not listed in the Lens Database, you will have to create a new lens ID. To do this, pick an unused ID number between 2000 and 3999 and enter the relevant information, mimicking the format shown in other lens ID entries. Sequence is unimportant, so a new lens can be added anywhere in the list of objectives. For more information, refer to the instructions at the beginning of the Lens Database file. Do NOT use spaces or tabs in the entry.

2.2. Adding the Lens information to Resolve3D:

2.2.1. In the main *softWoRx* window, select **Utilities > Revise Microscope Configuration.**

2.2.1.1. When prompted, type in the root password.

IMPORTANT: When modifying system files, Do NOT use tabs or other unusual characters. Spaces should be used to separate items for clarity.

2.2.2. Find the section titled: Microscope Specifications.

2.2.3. Increase the number of lenses (`MS_Number_Lenses`:) by 1.

2.2.4. Add the desired name of the objective to the list of lens names (`MS_Lens_Names`:). For example, 100xoil, 60xwater.

2.2.5. Enter the calculated pixel size(s) for each available camera in the appropriate position as indicated below:

`MS_Pixel_Size_1`:	Coolsnap HQ2
`MS_Pixel_Size_2`:	Cascade 2 EMCCD (conventional mode)
`MS_Pixel_Size_3`:	Cascade 2 EMCCD (EM mode)

NOTE: Pixel sizes 2 and 3 should be the same. If your system does not have an EMCCD camera, ad 0.1 for the MS_Pixel_Size_2 and MS_Pixel_Size_3.

2.2.6. Enter the lens ID number (`MS_Lens_ID_Numbers:`).

2.2.7. Save changes and Exit.

NOTE: In versions of *softWoRx* older than 3.7.0, you will have to enter the lens NA as well.

2.2.8. Restart *softWoRx*.

3. Collecting a Point Spread Function

3.1. Settings:

3.1.1. Make the adjustments listed in the table below:

Collecting a Point Spread Function	
Beam Selector	Eye
% Transmission	100%
EX Shutter	EX
Image Size	1024x1024
Bin	1x1
Stage	Repeatable Slide Holder

3.1.2. If your system is equipped with DIC components, make sure that the Principal Prism Slider (located under the stage) is in the appropriate position.

NOTE: The slider should be in the position in which it is left for regular image collection. For example, if most experiments are run with DIC reference images, the slider should be left in for PSF collection, however, if experiments are set up without DIC components, pull the slider out.

3.1.3. Make sure the appropriate objective is selected from the lens dropdown menu in the Resolve3D window.

3.2. Mounting a bead slide:

3.2.1. Open the oil calculator tool by selecting the **Info** button in the Resolve3D window.
NOTE: If you are using an air or water objective, skip to step 3.2.4.

3.2.2. In the Optical Conditions section of the Lens Information window, enter all relevant information: Distance from Coverslip to Specimen (μm), Temperature (^oC), Specimen Refractive Index and Coverslip Thickness (μm). Note the Recommended Refractive Index.

3.2.3. Select the oil with the Recommended Refractive Index and place one drop carefully onto the lens of the objective.

3.2.4. Place the bead slide (included in your Applied Precision Calibration Kit) coverslip down into the repeatable slide holder.

3.2.5. Rotate the eyepiece filter wheel to a channel appropriate for the bead slide.

3.2.6. Open the excitation shutter from the keypad and focus on the beads through the oculars.
NOTE: There are both large and small beads on each bead slide. The large beads are used to find the focal plane but the small (sub-resolution) beads should be used for PSF measurement.

3.2.7. Looking through the oculars, move to an area on the slide that is sparsely populated with small beads (the idea is to isolate one small bead in a small field of view).

3.2.8. Switch the beam selector to the camera and acquire an image. Make sure the Data Collection window is fit to screen  .

3.2.9. Use the centering tool  to center a small, bright and uniformly shaped bead in the image window.

3.2.10. Reduce the image size to 128x128. If necessary, use the centering tool again to center the bead.

3.2.11. Move through Z using the up and down arrows in the Resolve3D Window to find the plane of maximum intensity. When the plane of maximum intensity is found, mark this point.

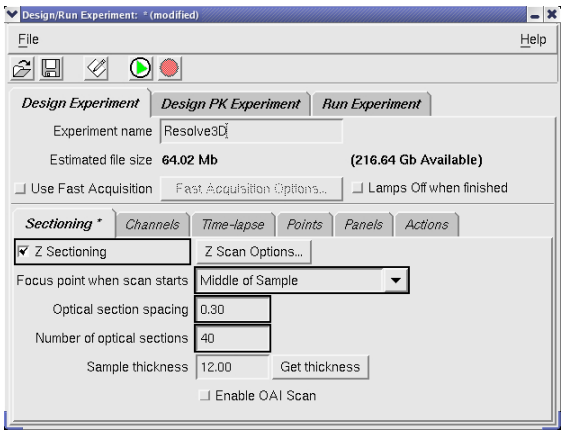
3.2.12. Adjust the exposure time to achieve a maximum intensity of about 2000 counts.
NOTE: If exposure time must be longer than 0.500sec in order to achieve this intensity, return to step 3.2.7 and find a brighter bead.

3.3. Setting up and running the experiment:

3.3.1. Return to the point marked in step 3.2.11.

3.3.2. Click on the **Experiment** button in the Resolve3D window to open the Design/Run Experiment window.

3.3.3. Set up the Sectioning tab as shown below:



3.3.4. In the Channels tab, click on the box to activate the first channel and then select the correct Ex Filter from the drop-down menu. Make sure the EM filter, %T and exposure time are loaded correctly from the Resolve3D window.

3.3.5. Click on the **Run Experiment** tab. Select **Yes** to save experiment design then select **OK** to save the experiment macro as the default file name.

3.3.6. Save images as: LensName_OilRI (example: 60xOil_1516RI).

3.3.7. Click the green play button to run the experiment.

3.4. Examining experiment file to determine degree of oil matching:

3.4.1. Scroll up and down through Z in the image window. Does the spread of light seem even in both directions?

NOTE: If the spread of light is difficult to see, open the Scale Image window and change the gamma from 1 to 0.3. The gamma is the last value on the **Display Min/Max/Exp** line.

3.4.2. In the image window, go to **Tools > Orthogonal Viewer**.

3.4.3. In the orthogonal viewer window, select **Options** and make sure that **Cover Slip At Bottom/Right** is selected.

3.4.4. Click on the green dotted lines and drag them to the center of the bead in order to view the spread of light from the center of the sphere.

3.4.5. Is the spread of light even above and below the focal plane? (See Figure 2.)

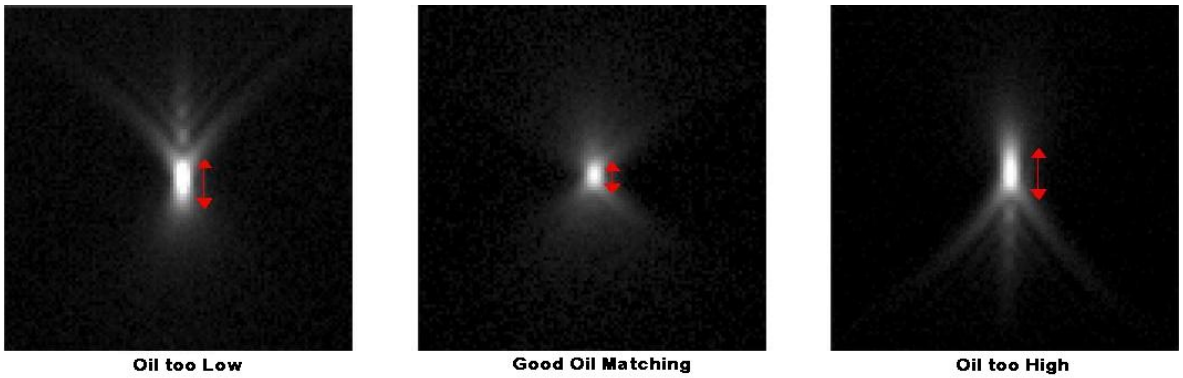


Figure 2. View of a bead in the orthogonal viewer demonstrating good and poor oil matching.

3.4.6. If oil matching looks good according to the criteria in 3.4.1 and as illustrated in Figure 2, use this oil to collect the experimental point spread function. If it does not, remove and clean the bead slide and clean off your objective. Adjust the oil up or down and repeat steps 3.2 – 3.4 until the optimum oil is found.

TIP: Compare the image at the bottom left-hand side of the orthogonal viewer to those in Figure 2. If the spread of light is heavier above the focal plane, your oil RI is too low and you should use oil with a higher RI. Conversely, if the spread of light is heavier below the focal plane, your oil RI must be adjusted down.

3.5. Collecting the Experimental PSF:

3.5.1. Finding and isolating a bead:

3.5.1.1. Using what was determined as the optimum oil, focus on the bead slide through the oculars and find a sparsely populated area.

3.5.1.2. Switch the Beam Selector to the camera and acquire an image. Use the centering tool  to center a small, bright and uniformly shaped bead in the image window.

3.5.1.3. Adjust the image size to 128x128.

3.5.1.4. Move through Z to find the plane of maximum intensity. When the plane of maximum intensity is found, mark this point.

3.5.1.5. Adjust exposure time to achieve a maximum intensity of about 2000 counts. **NOTE**: If exposure time must be longer than 0.500sec in order to achieve this intensity, return to step 3.5.1.2 and find a brighter bead.

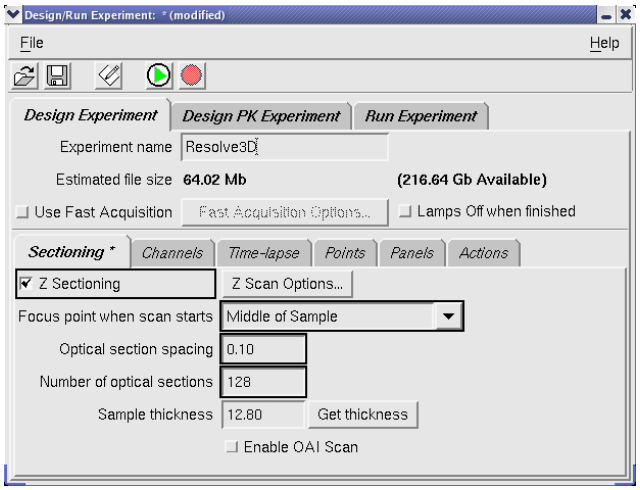
3.5.2. Setting up and running the experiment:

3.5.2.1. Return to the point marked in step 3.5.1.4.

3.5.2.2. In the Resolve3D window, select the **Settings** button. In the Resolve3D Settings window that opens, select the **Imaging** tab and change the **Frames to Avg** to 4. Select **Save Settings** then select **Done**.

3.5.2.3. Click on the **Experiment** button in the Resolve3D window to open the Design Experiment window.

3.5.2.4. Set up the Sectioning tab as shown below:



3.5.2.5. In the Channels tab, select the correct Ex Filter from the drop-down menu. Make sure the EM filter, %T and exposure time are loaded correctly from the Resolve3D window.

3.5.2.6. Click on the Run Experiment tab. Select **yes** to save experiment design then select **ok** to save the experiment macro as the default file name.

3.5.2.7. Save images as: LensMagnification_NA_psf (example: 60X_140_psf).

3.5.2.8. Click the green Play button  to run the experiment.

3.5.2.9. When the experiment is done running, move the file and the log file to /usr/local/softWoRx/data.

4. **Creating an OTF File**

4.1. From the main *softWoRx* window, select **Conversions > Convert PSF to OTF**. Select the **PSF File** button. In the window that opens, navigate to the /usr/local/softWoRx/data folder and select the correct PSF input file. Click **Do It**.

4.2. The new OTF file will have the extension .dv_otf. Copy the file from /usr/local/softWoRx/data to /usr/local/otf and rename it in the following format: LensMagnification_NA.otf (example: 60X_140.otf)

NOTE: The system is set up with OTF files for the existing objective lenses and it is best not to overwrite these files. If a lens has been replaced, rename the old OTF file before overwriting it.

5. **Modifying softWoRx to Associate Experimental OTF with an Objective**

5.1. Adding the OTF file to the system.swrc file will set up automatic entry of the OTF for the deconvolve function (i.e. when the lens is properly selected during an experiment, the correct OTF file will be loaded as the default in the Deconvolution window).

5.2. Open the Data Folder and navigate to /usr/local/softWoRx/config/ .

5.3. Find the system.swrc file. Right click on the filename and rename it with today's date: system_mmddyy.swrc .

5.4. Open the system_mmddyy.swrc file.

5.5. Find the section labeled "Lens/OTF matching".

5.6. Add the lens and OTF file information in the following format:

```
LENS_<lensIDNumber>_OTF    LensMagnification_NA.otf
```

Example:

```
LENS_10403_OTF      40X_075.otf
LENS_10602_OTF      60X_140.otf
LENS_10603_OTF      100X_135.otf
```

NOTE: Use spaces (NOT tabs) to separate lens and OTF file information.

5.7. Go to **File > Save As** and save the modified file as system.swrc .