


## 1 – Turn on the computers:

- Power on the monitor, camera and SSI (if 7-color).
- Power on the ICMIC and workstation. The order in which they are powered on does not matter.
- Power on the environmental chamber (if installed).

## 2 – Login and Initialize the system:

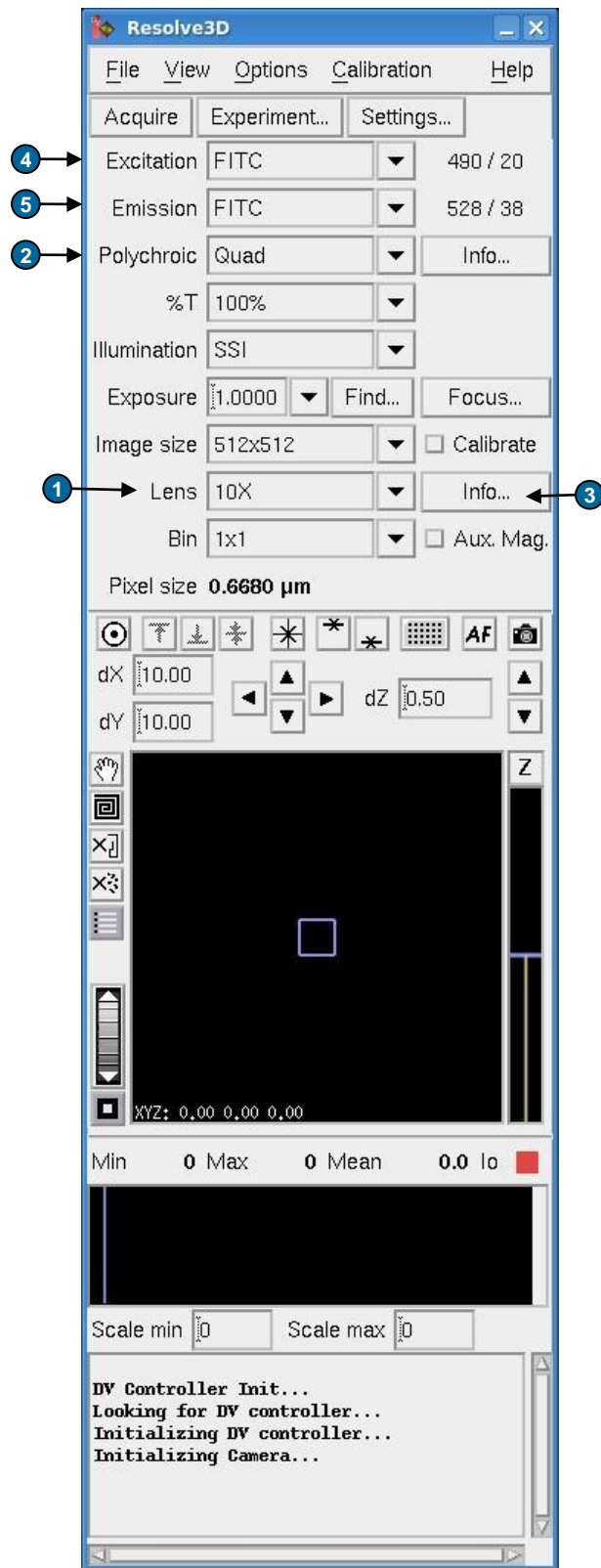
- Type in username and password. (default: User: worx Password: [system serial number]).
- Open softWoRx.  (icon may be located on desktop, in task bar, or in the start menu under the Applied Precision folder).
- To startup the microscope, in softWoRx select File -> Acquire Resolve3D.
- Follow the on-screen instructions.

## 3 – Mount your sample:













- Make sure the appropriate objective is selected in the lens area ❶ of Resolve3D.
- Rotate the dichroic turret to the proper position. If a motorized turret is installed, select the desired dichroic in the polychroic section of Resolve3D. ❷
- Select the Info button ❸ in the Resolve3D window. Enter information under Optical Conditions and note the Recommended Refractive index (RI).
- Place one drop of oil (with recommended RI) on Objective or Coverslip. Mount slide coverslip down in repeatable slide holder.

## 4 – Focus on sample through Oculars:



- Bring objective up to sample by rotating coarse focus knob towards you just until you see the oil spread.
- Rotate Eyepiece filter wheel to an appropriate channel.
- Observe the filter monitor window to ensure the proper filters are in position. If not, adjust the EX ❹ and EM ❺ filters accordingly.
- Rotate the port selector knob (located on the front of the microscope base) to the eyepiece.
- Open the EX Shutter from the Joystick Keypad.
- Look through the oculars and rotate the fine focus knob to focus on your sample.
- Use the joystick to scan your slide in x and y to find a region of interest.
- Close the EX Shutter from the Joystick Keypad.

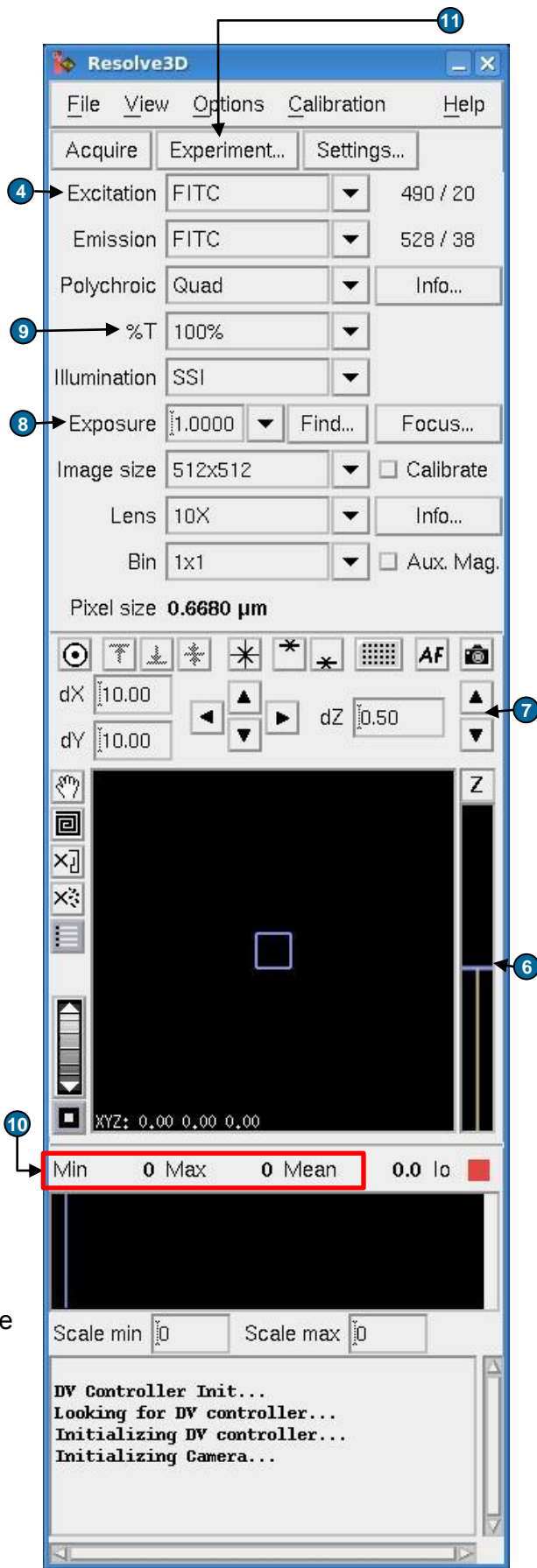


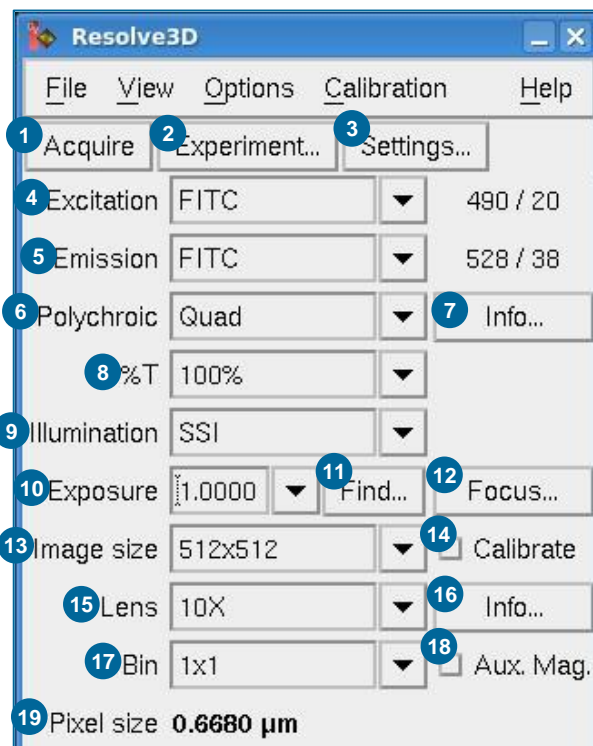
**5 – Set up experimental conditions:**

- Rotate the port selector knob to the camera.
- Acquire an image.  In the image window, select fit to screen.
- Use the Centering Tool  to center the specimen in the image window.
- Find focal plane (location of highest max intensity) using Z bar  or Up and Down arrows  in Resolve3D.  
**NOTE:** it may be necessary to lower exposure time if image is saturated (max intensity of 4095).
- Adjust exposure time  and %T  (Neutral Density) to achieve a max intensity  between 2000-2500 for fixed samples or 3-5 times the background (min intensity) for live samples.
- Switch EX filter  to the next channel and find appropriate exposure time and %T. Repeat for all channels that will be utilized in your experiment.
- Use the Z bar  to scroll up until you reach the top of your sample. Mark the top.   
**NOTE:** You should scroll up until the structures that you are trying to see in your experiment are just slightly out of focus.
- Use Z bar to scroll down until you reach the bottom of your sample. Mark the bottom. 
- Return to the middle of your sample. 

**6 – Design and Run Experiment:**

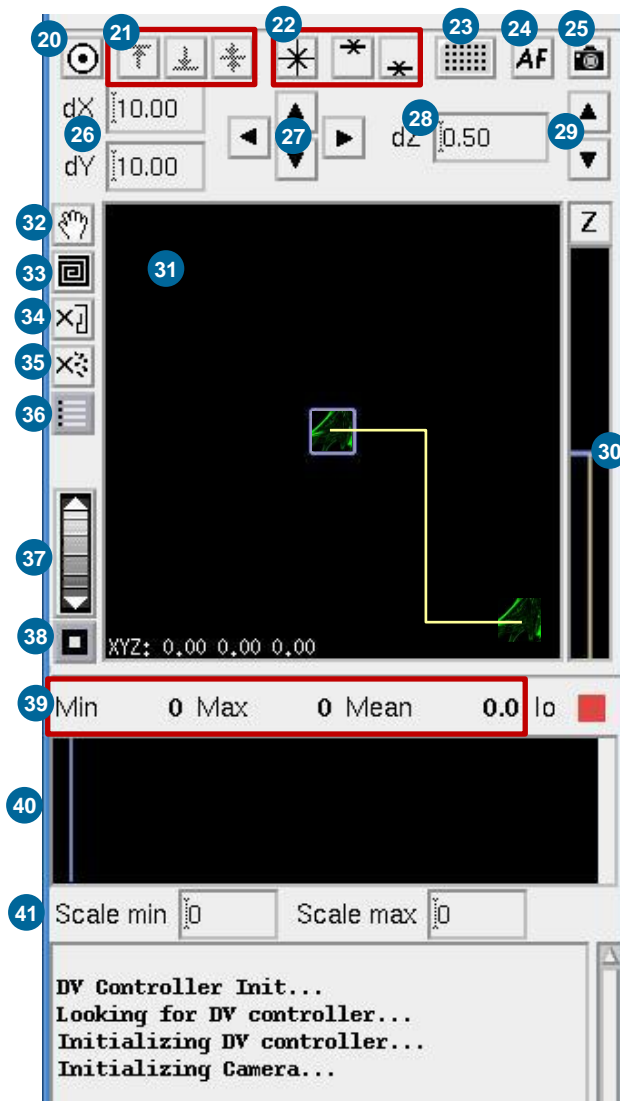
- Select the Experiment button  in the Resolve3D window.
- Under the Z Sectioning tab, select the Get Thickness button. This will load the Z thickness you set in Section 3. Make sure that you have elected to start from the Middle of the Sample.
- Under the Channels tab, activate the number of channels you wish to use in your experiment. Load the channels using the EX Filter drop-down menu.
- Select the Run Experiment tab. When prompted, select “OK” to save the experiment macro and then “Yes” to save the macro as the default filename (Resolve3D.exp).
- Select the Settings button to change where your image file is saved.
- Enter an Image File Name.
- Select the Play Button. 





1. Acquires an image.
2. Opens the experiment designer window.
3. Opens the Resolve3D settings window.
4. Excitation (EX) wavelength.
5. Emission (EM) wavelength.
6. Polychroic position (if motorized turret installed).
7. Opens polychroic information window (if motorized turret) installed.
8. Percent transmission (equivalent to neutral density filter).
9. Illumination type (SSI, Trans, Laser, etc.). (Also called EX shutter).
10. Exposure time (seconds) can be selected with dropdown or typed in.
11. Finds the optimal exposure time for a specified max intensity.
12. Opens focus assist/ coverslip finder (if UltimateFocus installed).
13. Specifies image size (must be  $\leq 512 \times 512$  if binning larger than 1x1 is to be used).
14. When selected, will apply calibration file (if it exists). A calibration file must be created for each combination of image size, channel (EX, EM, and polychroic), objective, and camera used.
15. Objective Lens, must be changed when changing objective.
16. Displays lens information, including optical conditions and recommended oil refractive index.
17. Binning size (must be 1x1 if  $> 512 \times 512$  image size is used) signifies how many pixels on the camera translate to each pixel in the image.
18. Auxiliary magnification should be selected if the auxiliary magnification slider (located on the right side of the base below the polychroic turret) is being used.
19. Shows the pixel size in micrometers.

20. Centering tool – select the tool then select a spot in the image that should be in the center. The stage will move to the appropriate location.
21. Visit Z positions: visits Top, Bottom, and Center of z selection, respectively.
22. Mark stage position: marks Stage Coordinate, Top of Sample, and Bottom of Sample, respectively.
23. Microtiter plate viewer (if microtiter stage installed).
24. Software (contrast-based) AutoFocus.
25. Acquires an image.
26. Step size for X and Y movement when arrows (27) used.
27. X and Y movement arrows.
28. Step size for Z movement.
29. Z movement arrows.
30. Z stage position – visual indicator of z stage position (blue bar) and marked top and bottom of sample (yellow bars).
31. Stage Window – visual indicator of XY stage position (blue box), stage motion trails (yellow lines) and thumbnails of sample, shows XYZ stage coordinates in bottom left corner.
32. Hand tool – pans the stage view (31).
33. Spiral mosaic – takes a series of images in a spiral to give an overview in thumbnails of the sample in a single channel.
34. Clears stage trails from stage view area.
35. Clears thumbnails from stage view area.
36. Shows point list window.
37. Zoom – zooms in and out stage view area.
38. Zoom Factor 1 – returns stage view area to 1x zoom.
39. Image intensity values: Min, Max, Mean.
40. Histogram – shows histogram of intensity values.
41. Sets min and max values of histogram.



## Experiment Designer

### ▪ Sectioning Tab:

#### ▪ *OAI (Optical Axis Integration) Scanning:*

- Select the box next to OAI scan to run an experiment that will collect a 2D projection of the 3D data contained within the Z thickness specified.
- Before deconvolving these files, be sure to select the Deconvolve Projections box in the Deconvolution tool.

### ▪ Channels Tab:



- To add a reference image, check the box and select where in the z-stack the image will be taken, in what channel and with which exposure settings.
- If DIC is to be used, select POL for the filter sets, and TRANS for the exposure source. Make sure the prism slider is in place and the contrast adjusted to the desired amount.

### ▪ Time Lapse Tab:

- Activate the time lapse tab and enter the time between points and total time you wish to collect.
- *Tracking and focus options:*
  - Cell tracking should be turned on if observing an isolated cell that is expected to migrate in x and y. It will keep the cell within the field of view by moving the stage to compensate for cellular movement.
  - UltimateFocus maintenance will use the infrared laser to maintain the focus set at the start of the experiment.
  - Image based Autofocus will use contrast in fluorescence (or transmitted) light images to find the focal plane.
- *Note:* If there is significant time between points of your time lapse, you may want to select the “Deconvolve during experiment” option in the Run Experiment tab.

### ▪ Point Visiting Tab:

#### ▪ *Find and refine the desired points:*

- Mark points using either the bottom right button on the Joystick keypad or the  button in Resolve3D.
- From the Resolve3D window, open the Points List.  Without adjusting the microscope focus knob, visit each point and find its focal plane. Select the Replace Point button in the points list to save this Z position.

#### ▪ *Set up the experiment to visit the points :*

- Activate the Points tab, list the points you would like to use in your experiment (i.e. 1-3, 5,10-14).
- The experiment will apply all Z sectioning, Channels and Time Lapse settings to all points selected.
- Both UltimateFocus and image based auto focus options can be defined if desired.




## Experiment Designer

- Panels:
  - A series of images that can be stitched together to form a single large image is a panel experiment.
  - *Consider the desired image characteristics:*
    - If the final image is potentially large, binning may be used to reduce the file size.
    - Special consideration should be taken if the image will be deconvolved after acquisition
      - The overlap value listed in the Panels tab of the Experiment Designer must be at least 2x the border roll-off of the image. Follow the steps below to check what the border roll-off value is:
        - Select the correct image size, bin value and objective in the Resolve3D window.
        - In the Resolve3D window, select File > Snapshot.
        - Activate one channel and select Do It. (Note: it is not necessary to have a sample on the scope or the correct channels selected for this portion.)
        - In the main softWoRx window, select Process > Deconvolve.
        - Drag the window ID number for the snapshot into the Input field in the Deconvolution Window.
        - In the Deconvolution Window, select the More Options button.
        - In the More Options Window look for the field labeled Border Roll-off.
        - Note the value of the border roll-off and double it for the Overlap value during panel acquisition.
    - Selecting “Get Start” or “Get End” will set the current stage position as the start or end of the panel. (The image will be scanned from upper left to lower right).
- Actions:
  - During a time-lapse experiment, the user has the flexibility to add any number of actions to specialize the experiment.

## 1 – Experiment File Location

- All experiment files are saved in the Data Folder (Data1). This folder can be opened from the Main softWoRx window by selecting the Data Folder button or by double clicking on the Data1 folder on the Desktop.

## 2 – Image Processing

- General Processing Tips:
  - Loading files into processing tools. First, open the tool, then do one of the following:
    - Drag and drop the window ID number of the file into the Input line of the tool.
    - Type the window ID number of the open image file into the Input line of the tool.
    - Drag and drop the image file icon from the Data folder into the Input line of the tool.
  - Many tools have a Details button. This is where you can choose to process only a subset of your data by selecting only certain Z sections, Time Points, etc.
  - Many tools also have a Select Region button. This is where you can select only a particular x-y area to process. select the Select Region button then draw a box in your image window. The reset button will allow you to erase a selected region.
  - Not all tools automatically save their output. You will be prompted to save unsaved files before closing.
  - Select the  button to open the image histogram (or use the small histograms located on the top of the image window). This will adjust display scaling but will not alter your experimental data.
- Deconvolution: Select Process > Deconvolve. All other information is loaded from the raw image file. Default settings are adequate for the majority of applications. Select Do It.
- Quick Projection: Select View > Quick Project. This will project your 3D data into a 2D format by either Max Intensity, Average or Sum method.
- Volume View: Select View > Volume Viewer. Select the Rotation you prefer then select Do It.

## 3 – Save As

- Movie: In the image window, select File > Save as Movie. Select the preferred file format (MPEG, AVI or Quicktime), and Frame Rate then select Preview. When the speed of the file looks ok, select Do It.
- Tiff: In the image window, select File > Save as Tiff. Decide whether you would like the Tiff images scaled or unscaled by selecting or deselecting the “Scale Using Min/Max/Exp Values Below.” Select the appropriate Output Size then select Do It.  
**NOTE:** softWoRx will make a separate Tiff file for every z section and time point in your experiment. Select a subset in Details if you wish.
- JPEG Snapshot: In the image window, select File > Save as JPEG. Select Do It.  
**NOTE:** Do NOT save as JPEG if you hope to quantify your data.