STANDARD OPERATING PROCEDURE

Deltavision Elite Deconvolution Microscope

PURPOSE

This document describes the operational setup and procedures for the Deltavision Elite Deconvolution microscope (Deltavision). This document offers users, particularly beginners, a brief step-by-step instruction on how to take images using the Deltavision microscope with its acquisition software Softworx 6.5.2.

The Deltavision microscope is a high-end wide-field fluorescence inverted microscope featuring a software-based restorative deconvolution process. The system is equipped with optimised solid illumination light with fast excitation switching (microsecond) that allows fast and bright multicolour 3D live cell imaging resulting in reduced specimen fading and photobleaching. Dual cameras (CoolSnap HQ and EMCCD) provide high resolution and high sensitivity options. Features include speed, sensitivity and high-resolution of images making it suitable for small/thin and faint fluorescence samples as well as living cells.

SCOPE

The procedure covers preparing the system, start-up & basic acquisition, advanced acquisition options including sectioning (Z stacking), multiple channels, time lapse, multiple positions etc., as well as basic image visualization & processing.

Users are to refer to the full manual provided by the vendor for more detailed operational instructions. The full manual can be found on the WRH Cell Imaging website.

SAFETY

The operator must exercise caution during initialisation of the stage. Never put fingers inside the incubator while the stage is moving or initialising.

Safe operation of the instrument requires the awareness of risks e.g. biological spills, glass cuts, fatigue and gesture discomfort/injury etc. Refer to the "WIMR-SWP-WHS-GEN-16.01 Use of fluorescence and laser microscopes" for details:
https://sydneyuni.atlassian.net/wiki/spaces/WIF/pages/768016621/Manuals+Protocols.

Acquisition of samples on the Deltavision microscope must only occur after the approval of an associated project in PPMS. Any projects to be run on the Deltavision involving hazardous chemicals must have appropriate approval. OGTR requirements for safe work in a PC2 laboratory apply.

TRAINING / COMPETENCIES

All personnel require training prior to independent operation of the instrument. Training is conducted by facility staff with competency demonstration necessary before authorisation.

Competency is assessed via demonstration of independent instrument operation, in conjunction with verbal explanation of all aspects of operation and troubleshooting common faults. After training is carried out, a training competency quiz is to be completed and passed before instrument access is authorised by imaging staff. All instrument operation is to be conducted by trained operators.

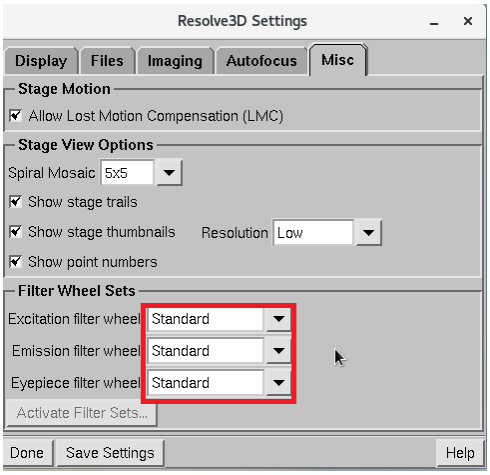
EQUIPMENT & SUPPLIES

The Deltavision microscope is located in J2.12A, Level 2 of WIMR.

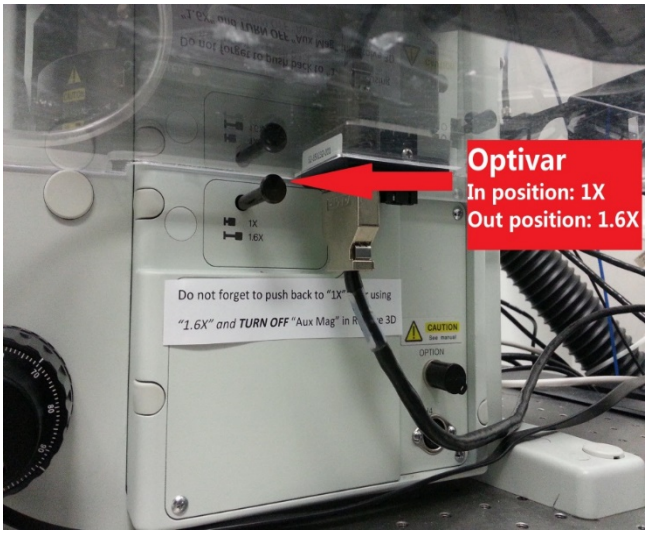
PROCEDURE

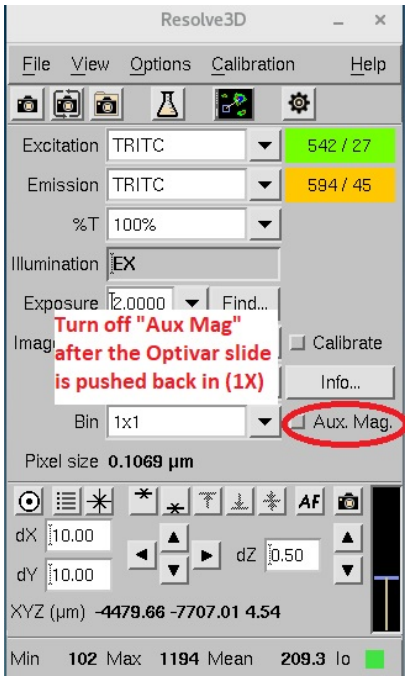
Important notes. Please keep the following default set-ups of the microscope in position after your use:

- System should always be left on (do not power off unless you are advised otherwise by the Imaging staff)
- Coolsnap HQ2 camera attached to the microscope (not the EMCCD)
- Filter wheel set: Standard set (not the Live Filter Set). Also make sure in the "Settings" in Resolve 3D, the Standard filters are selected as shown below



- Dichroic mirror/filter set for the red channel: "1" Position ("2" is for Alexa 594)
- "1X" Optivar is pushed in (Pull out: "1.6X") and the "Aux Mag" is turned off in Resolve 3D





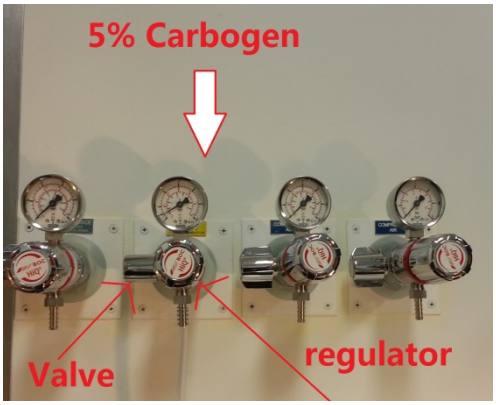
1. Start up

1.1 Turn on. The system should be left on all the time so no need to turn on any parts if you are just imaging fixed samples.

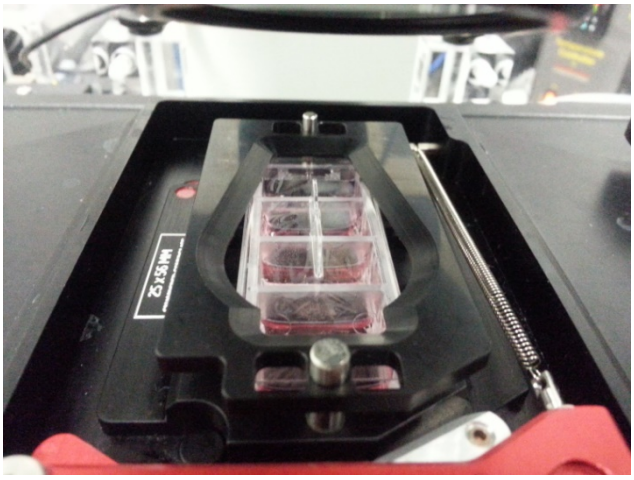
For live cell imaging settings, turn on additional components for CO2 supply and temperature control. **Note**: Warm up the weather station for at least 3 hours in advance to equilibrate the temperature and CO2 (Do not forget to place the appropriate oil in the Incubator so it will be warmed up as well).

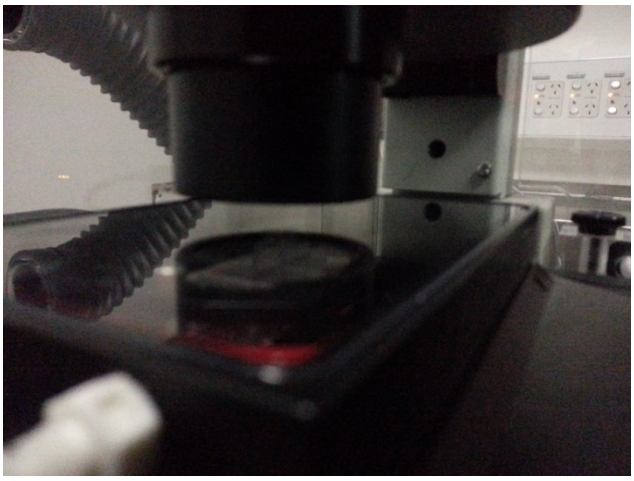
- Open the carbogen gas valve on the wall and then turn on the regulator clockwise to the marked line.

- Press the “Heat” button and choose “Hi” for fan speed of the Weather Station to blow the heated air into the incubator. Slowly adjust the flow till it reaches “3”.



- Set up the specimen like this (for beforehand warm up, you can use a used/empty chamber to seal CO2 leakage and later replace with your own sample container) (below left picture) and cover with the lid (below right picture).



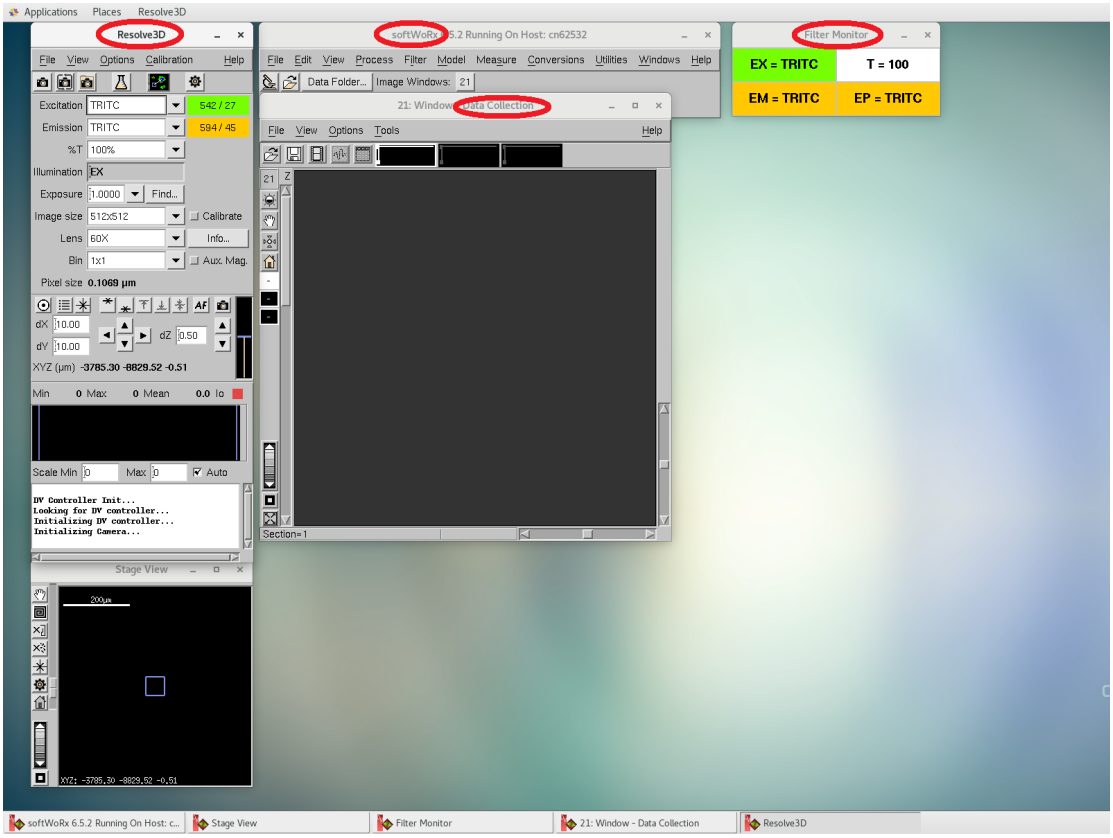


More information for live cell imaging settings is provided in “CO2 and temperature settings for live cell imaging with the Deltavision microscope”:
https://sydneyuni.atlassian.net/wiki/spaces/WIF/pages/768016621/Manuals+Protocols.

1.2 Log in to the Workstation

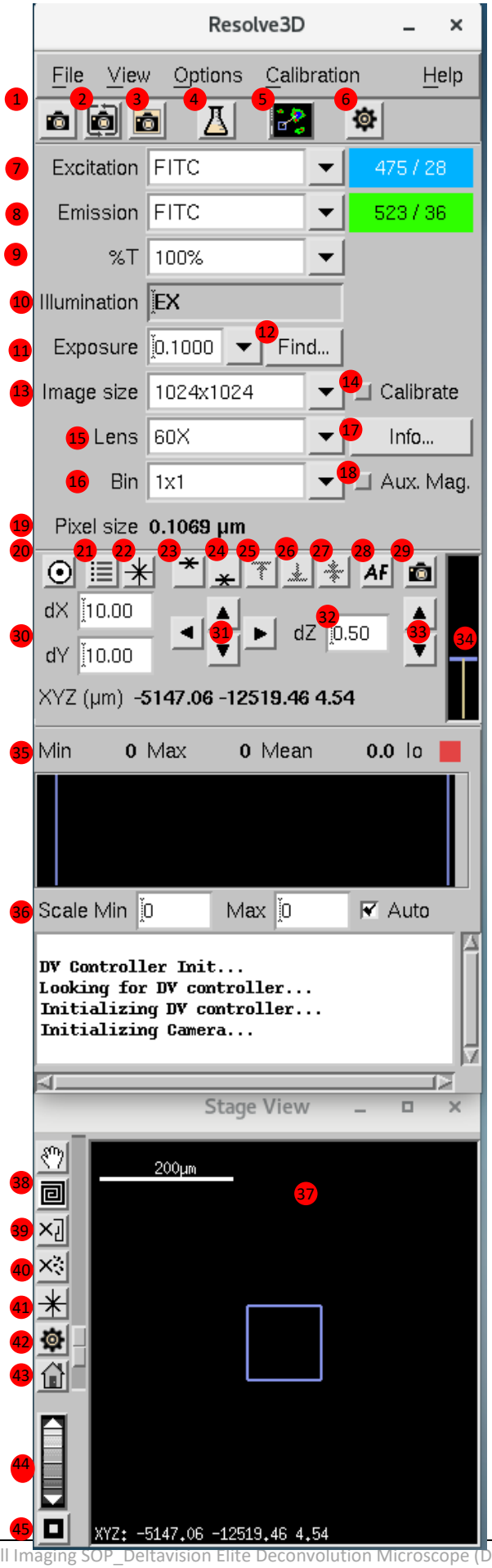
1.3 Start Softworx: “Applications”/”GE Healthcare”/”Start Softworx”.

1.4 Open up Resolve 3D and other components of Softworx: “File”/”Acquire”. Then the following 4 sub-windows will be brought up as shown below:
Resolve 3D: set up the microscope, imaging conditions and experiment (pre-acquisition)
Data collection: live window to view what’s being seen on the camera (live imaging)
Softworx: image viewing, processing and analysis (post-acquisition)
Filter monitor: display the eyepieces filters including excitation and emission as well as illumination intensity



2. Procedures

2.1 Resolve 3D features



3.1 Resolve 3D features

1. Acquire an image of the current channel
2. Continuous acquire images to display changes in the live window i.e. manual adjustment of the focusing knob
3. Save snapshot
4. Open the Experiment Designer window
5. Bring stage view window (37) to the front
6. Open the Resolve 3D Settings window
7. Excitation wavelength
8. Emission wavelength
9. Percentage illumination intensity
10. Illumination type (EX: fluorescence; Trans: transmission)
11. Exposure time (seconds): can be selected from drop down list
12. Find the optimal exposure time for a specific max intensity
13. Specify image size (must be ≤ 512X512 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, channels, objective and camera used
15. Objective lens. Must be changed when changing objective
16. Binning size (must be 1X1 if > 512X512 image size is used) signifies how many pixels on the camera translate to each pixel in the image
17. Display lens information including optical conditions and recommended oil refractive index needed to be used after inputting the required information
18. Auxiliary magnification should be selected if the auxiliary magnification slider is being used
19. Show the pixel size in micrometres
20. Centring tool: click on the tool then click on a spot in the image that should be in the centre. The stage will move to the appropriate location
21. Show point list window

22-14. Visit Z positions of Centre of Z selection (22), Top (23) and Bottom (24) respectively

25-27. Mark stage positions of Top (25), Bottom (26) and Stage Coordinate (27) respectively

28. Software-based or contrast-based Autofocus
29. Acquire an image of the current channel
30. Step size for X & Y movement when arrows (31) used.
31. X & Y movement arrows
32. Step size for Z movement when arrows (33) used
33. Z movement arrows
34. Visual indicator of Z position (blue bar: current stage position; yellow bars: marked top & bottom of sample
35. Image intensity values: Min, Max, Mean
36. Set min and max values of histogram
37. Stage window: visual indicator of XY stage position (blue box) and XYZ stage coordinates (bottom left corner)
38. Pan the stage view (37)
39. Clear stage trails from stage view area
40. Clear thumbnails of the sample in a single channel
41. Mark the current stage coordinate to add it to the Marked Point list
42. Settings (same as 6)
43. Restore the stage View to its original position
44. Zoom in and out stage view
45. Zoom factor 1: return stage view area to 1X zoom

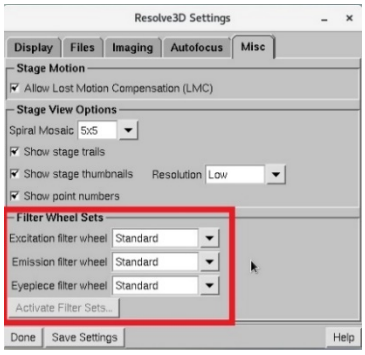
2.2 Preparation

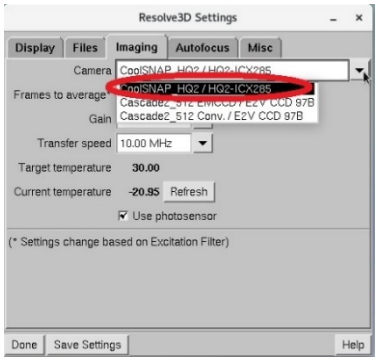
2.2.1 In Resolve 3D

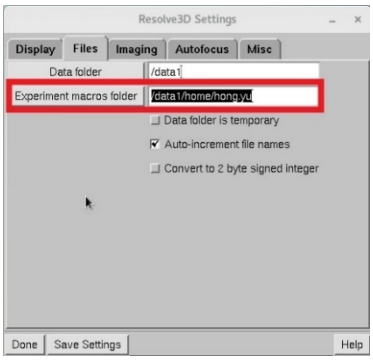
- Click "Settings" (6) and visit the following Tabs:
  "Misc" Tab: make sure filters are correctly selected in Resolve 3D settings and physically placed in position on the microscope.

  "Imaging" Tab: chose the camera suitable for your sample/imaging requirements. Coolsnap: high resolution and applies to most of applications. EMCCD: high sensitivity good for faint staining or to meet fast imaging requirement.

  "Files" Tab: users are required to follow:
  Data management policy: all data must be saved here (kept for 7 days before auto deleting: Data1/Home/"your folder". Your data will be transferred to the server instantly: Scientific Platforms\Cell Imaging\Deltavision\"your folder" (kept for 14 days before auto deleting). Your images can also be found on the Deltavision analysis computer (kept for 14 days before auto deleting): data



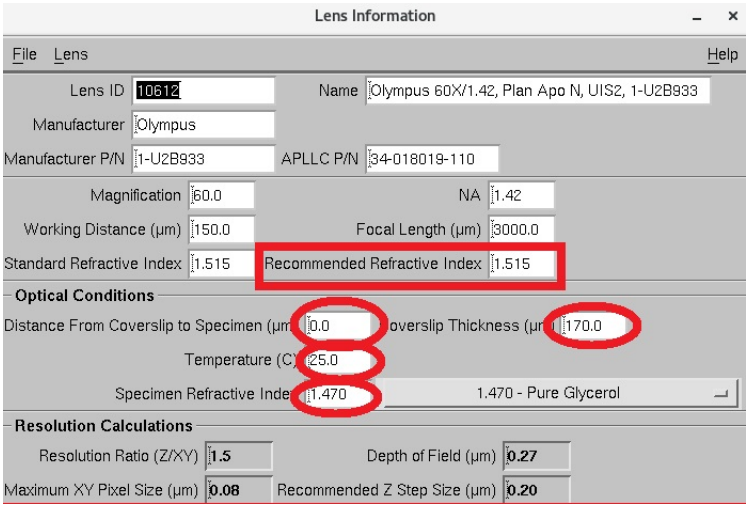




- Make sure an appropriate lens is selected in the lens area (15).
- Choose the Image Size (13).
- Click the "Info" button (17). Enter the information under current optical conditions including:
  "Distance from coverslip to sample" (you can start with an estimate)

  "Coverslip thickness" (i.e. 1.5# coverslip: 170 um)

  "Temperature" (reading can be found on the Weather Station metre)



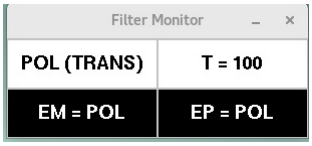
"Specimen refractive index". In the screenshot, it recommends 1.515 (as all the Refractive Indices of the provided oils end in even numbers so in this case you can start with either 1.514 or 1.516.

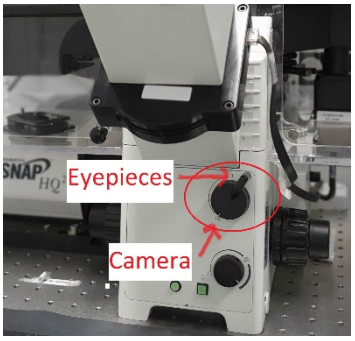
2.2.2 Get the microscope ready

- Turn the objective turret to select the lens. Objectives can be identified by the colours of the rubber band around the objective neck (refer to below table): 100X (purple), 60X (green), 40X (blue), 20X (empty).
- Make sure condenser position matching your objective (i.e. Position "3" for 40X as shown below)

Objective	Rubber band colour	Condenser position
20X	NA (empty)	3
40X	Blue	3
60X	Green	4
100X	Purple	5

- Clean the lens with provided 100% ethanol and cotton swabs.
- Place one drop of oil (with recommended RI) onto the coverslip (for fixed sample) or the objective (for chambered slides/coverslip when imaging live samples).
- Mount the sample (coverslip side down) in the slide holder.
- Rotate the eyepiece filter wheel to an appropriate channel. Make sure the Excitation and Emission filers are the same, i.e. for transmission (or BF) channel, Ex: POL; EM: POL.
- Rotate the port selector knob to the eyepieces





2.2.3 Focus on sample through eyepieces

- Bring objective up to sample by rotating course focus knob located on the front of the microscope base) towards you just until you see the oil spread.
- Open the shutter i.e. transmission shutter from the joystick keypad
- Look through the eyepieces and slowly bring the sample in focus by adjusting the fine focus knob.

  Note: special attention needs to be paid to avoid bringing the objective too high to push the slide off the sample holder.
- Use the joystick to scan your slide in X and Y to find a region of interest.

2.2.4 Check out 3 things

Koehler Illumination (Recommended to run this procedure once at the beginning of your booking session). Detailed instruction on Koehler Illumination procedure can be found on Imaging site page: https://sydneyuni.atlassian.net/wiki/spaces/WIF/pages/765397549/Tips+Tricks.

- Close the field diaphragm (at the top of the microscope where the light is coming in) so you can see its polygonal contour in the eyepiece
- Focus the edges of the brighter polygon-shaped contour by adjusting the condenser height (the knob on the right side of the microscope back pole).
  Note: you should not adjust focus knob to achieve the in-focus polygon edges!
- Centre the condenser by fiddling with two centering pins until the polygon contour locates in middle of field-of-view.
- Open the field diaphragm until it is just outside the edge of the field of view.

DIC

- Push the Polariser in (the silver part at the top of the microscope under the field diaphragm).
- Push the DIC Slider in (beneath the stage) and if necessary, twist the DIC Slicer to get a satisfactory DIC effect.

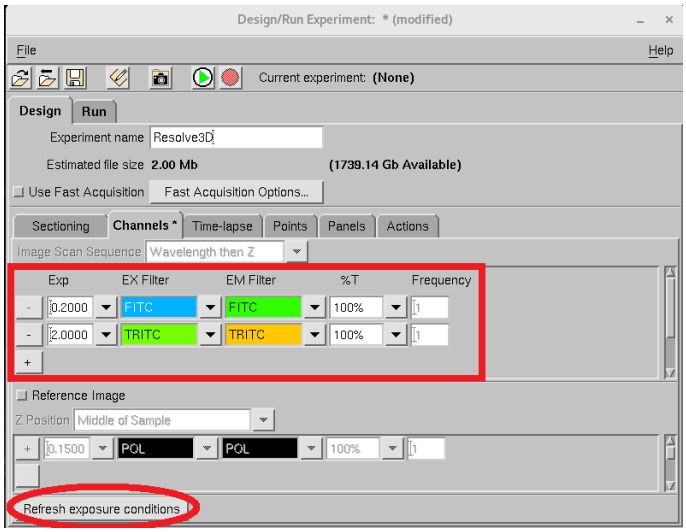
Fluorescence

- Open the fluorescence shutter
- Choose a filter to view your channel i.e. DAPI channel. Repeat this to view all fluorescence channels.
  Note: leave DIC slider and Polarizer out while imaging fluorescence. If DIC image is needed, it must be acquired separately.

2.3 Basic image acquisition

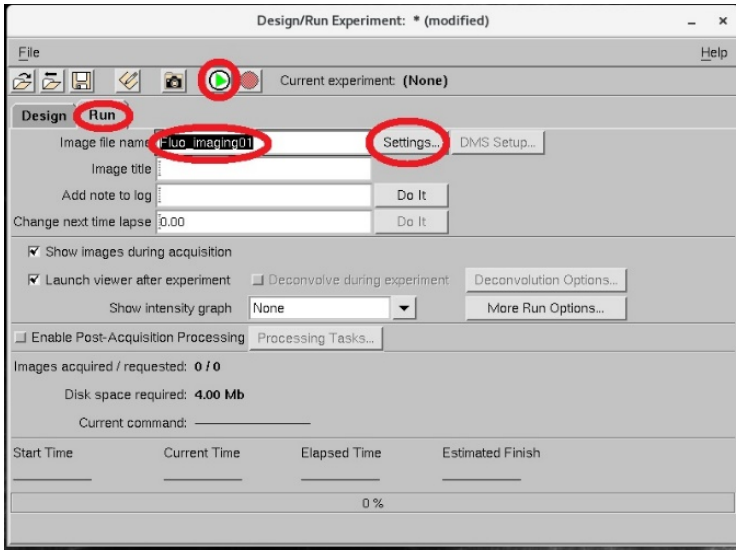
2.3.1 Set up the experimental conditions for all to-be-imaged channels

- Rotate the port selector knob to the camera.
- In Resolve 3D, "Acquire" an image (29) to view the current channel first in live window based on the current settings (illumination intensity exposure time etc, etc).
- Use the "Centring Tool" (20) to centre the specimen in the image window.
- If necessary, adjust focusing using either "AF" (autofocus 28) or Up/Down arrows (33) or manually adjust the focus knob on the microscope while "Continuous acquire" is turned on in Resolve 3D (2).
- If necessary, adjust exposure time (11) and illumination intensity (9) to get the right saturation for the image to achieve (if you can) "Max" 2000-2500 for fixed samples and 3-5 times the background ("Min" intensity) for live samples (35).
- Switch "Excitation" filter ( ) to the next channel (After you choose the "Excitation" filter in the drop down list, the corresponding "Emission" filter (8) will be selected automatically). Repeat the following steps to set up focusing, exposure time and illumination intensity etc.



2.3.2 2D multi-channel image acquisition

- Click "Experiment" (4) in the Resolve 3D. Go to "Channels".
- Click "+" to add channels you have just set up in the Resolve 3D (Section 2.3.1).
- IF DIC/BF is to be used, select POL for the filter sets.
- Load the channel settings by clicking "Refresh exposure conditions" in the Design/Run Experiment".
- Select "Run" tab. When prompted, click "ok" to save the experiment macro.
- Enter an image name (no space allowed in the name).
- Select the "Play" button (green arrow) to initialise image acquisition.



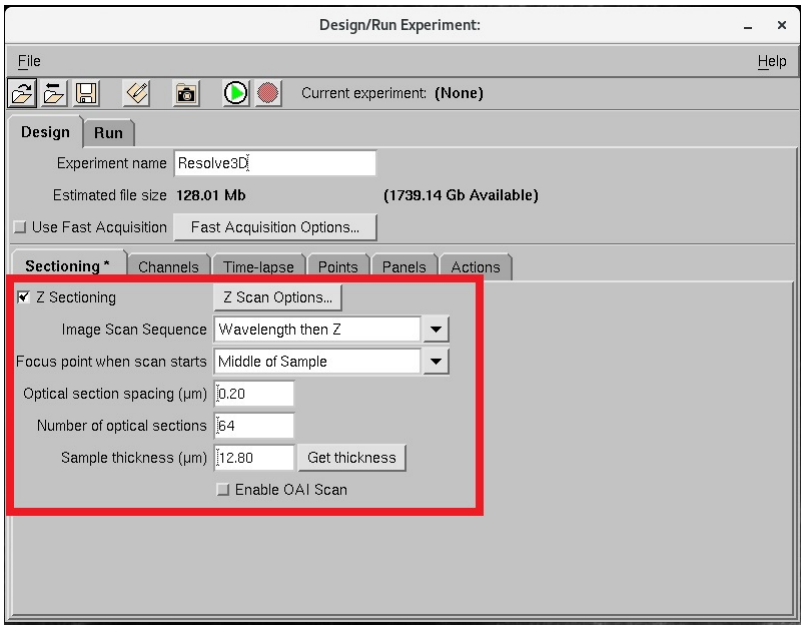
2.4 Z sectioning

2.4.1 Set up experimental conditions in Resolve 3D

- Set up channels as described in Section 2.3.1.
- In Resolve 3D, use the Z bar (34) or Z movement arrows (33) to scroll up until you reach the top of your sample. Mark the Top (25). Same way to scroll down until you reach the bottom of your sample. Mark the Bottom (26).

2.4.2 Design and Run Z sectioning experiment

- Click the Experiment button (4) in the Resolve 3D. Go to "Channels".
- Click "+" to add channels you have just set up in the Resolve 3D (Section 2.3.1).
- Click "Z Sectioning" tab. Optical section spacing: 0.1-0.3 um is okay. This Z step-size will determine your Z sampling rate. Clicking "select thickness" will load the Z thickness you set in Section 2.4.1.
  Note: Make sure that you have elected to start from the Z position of your sample i.e. Middle of the Sample in this case as shown below.
- Select "Run" tab. When prompted, click "ok" to save the experiment macro.



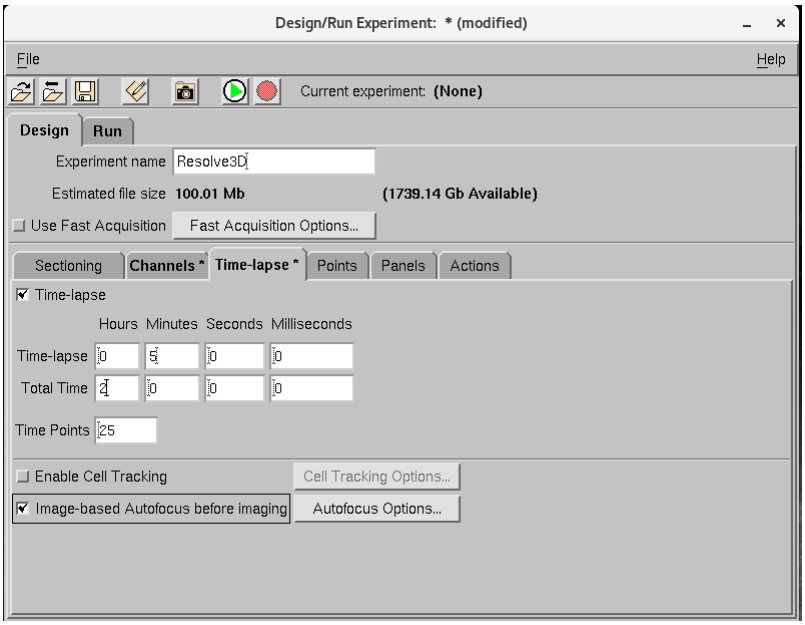
- Enter an image name (no space allowed in the name).

2.5 Time lapse

- Set up channels as described in Section 2.3.1.
- In "Experiment" designer, 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 tat is expected to migrate in X and Y. It will keep the cell withing the filed of view by moving the stage to compensate for cellular movement.

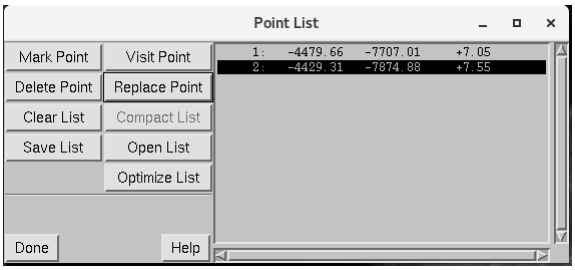
  AutoFocus will use contrast in fluorescence or transmission light images to find the focal plane.

  Note: if there is significant time between points of your time lapse, you may want to select the "deconvolution during experiment" option in the Run Experiment Tab.

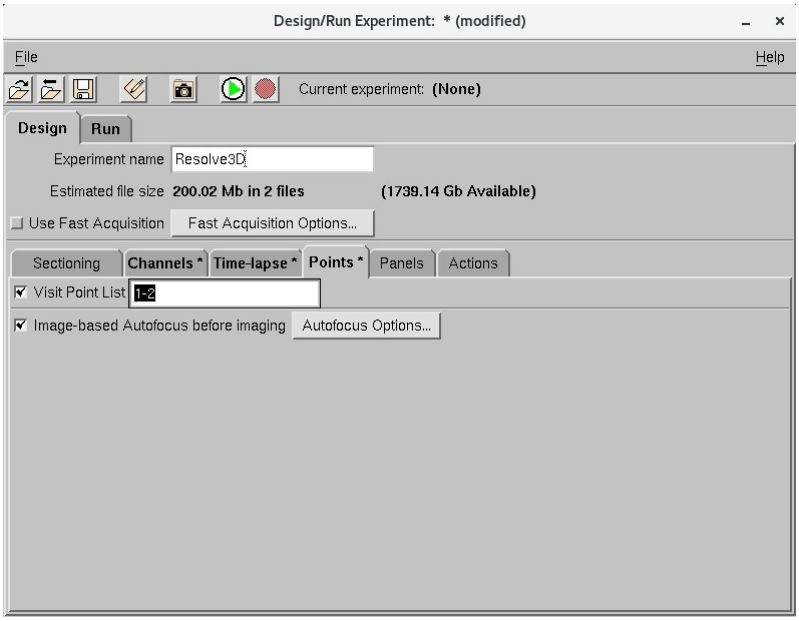


2.6 Points

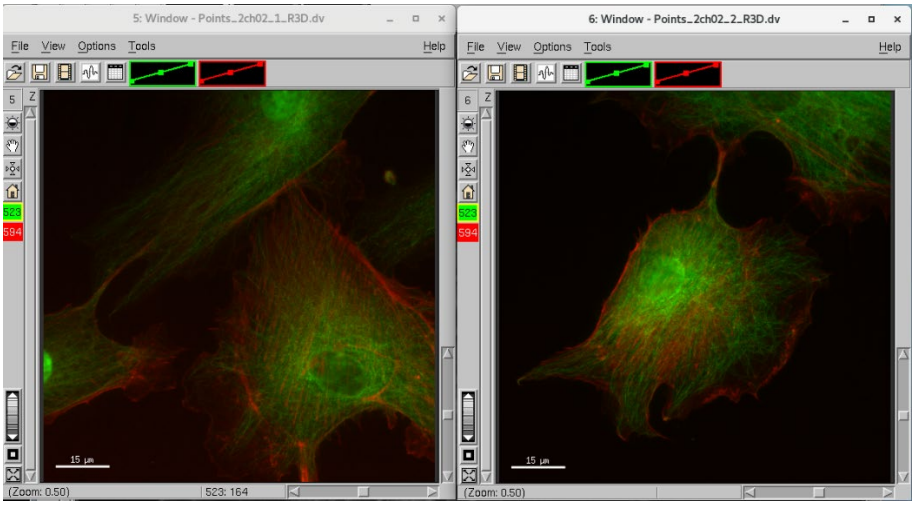
- Set up channels as described in Section 2.3.1.
- In "Experiment" designer, open the Points List in Resolve 3D (38). Mark points using either (41) or the bottom right button "Point Mark" on the Joystick Keypad. You can also use the joystick to move to a position of interest and click "Mark" in the Position list. Repeat this step to look for all interesting positions/points and add each point to the list.



- You may "Visit" each marked point later and refine its focal plane using Z movement arrows (33) (you should not physically change the microscope focus knob). Click the "Replace" button in the points list to save the Z position.
- Open "Experiment" and go to "Points" tab. List the points you would like to use in your experiment i.e. 1-3, 5, 10-14 etc.
- You may add "Z sectioning" (refer to Section 3.3), and/or "Channels" (refer to Section 3.2), and/or "Time Lapse" (refer to Section 3.4.1). Autofocus can be applied (refer to Section 3.4.1).



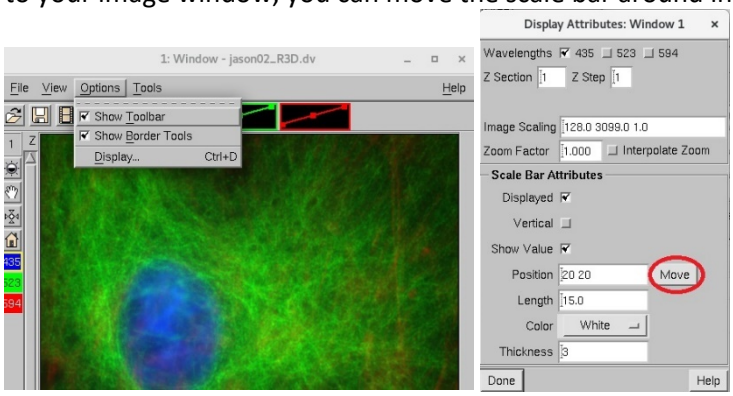
- The resulting images will be saved separately for each point (below shows two 2D images for two points for two channels).



2.7 Simple Post-acquisition processing and analysis

2.7.1 Add a scale bar

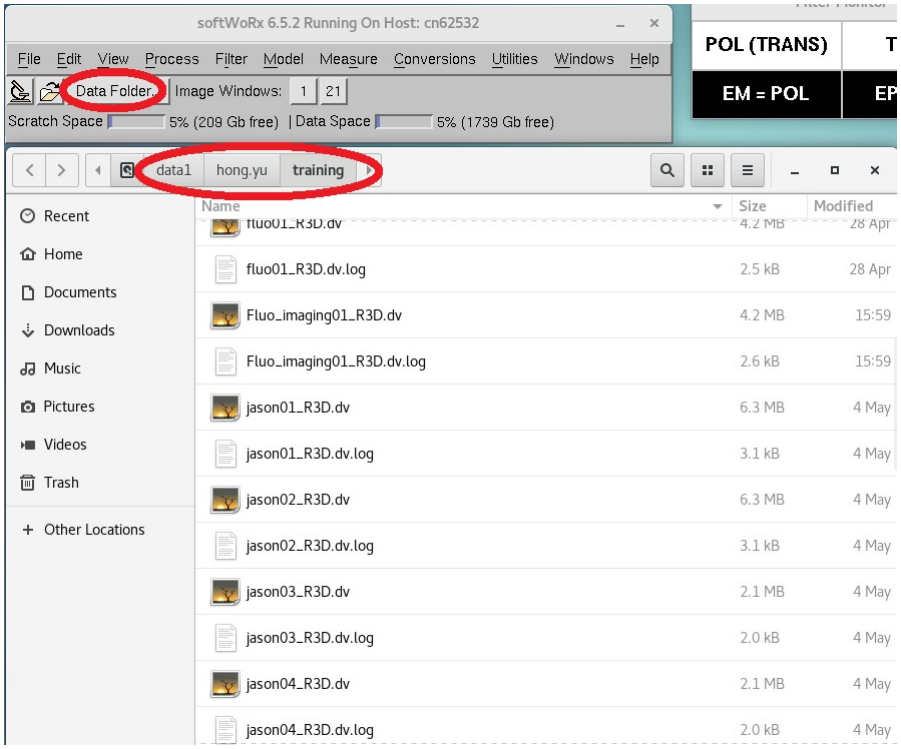
- In the image window, select “Options”/”Display” (as shown below in the left screenshot). You will then have the “Display Attributes” window popping up for scale bar settings. If you click “Move” and then go to your image window, you can move the scale bar around in the image.



2.7.2 Save data

2.7.2.1 location

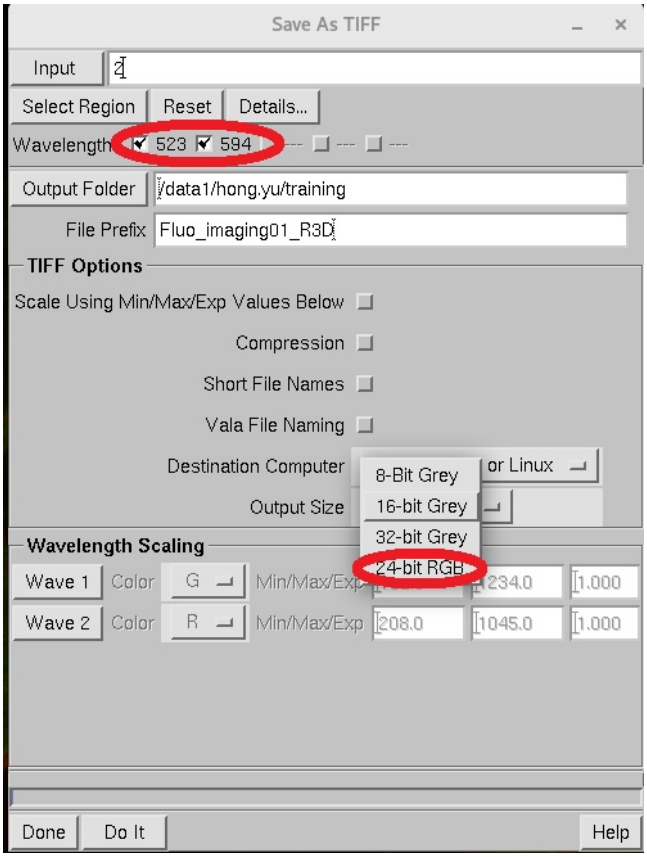
In Softworx”, click ”Data Folder”, your images should be saved here: ”Data1”/”your folder”.



2.7.2.3 Save as Tiff

- If you run image acquisition in “Experiment”, your images will be automatically saved to your defined folder in the natural format .dv.
- To save as Tiff. In the image window, Wavelength: Choose the channel(s): “435” represents DAPI Ch, “523” represents FITC/green Ch, “594” represents “TRITC/red Ch. If you need individual channel Tiff images, you need to save one at one time separately.

  Output size: either Grey scale or RGB for fluorescence images. For BF image, only grey scale format works (either 8 bit or 16 bit).



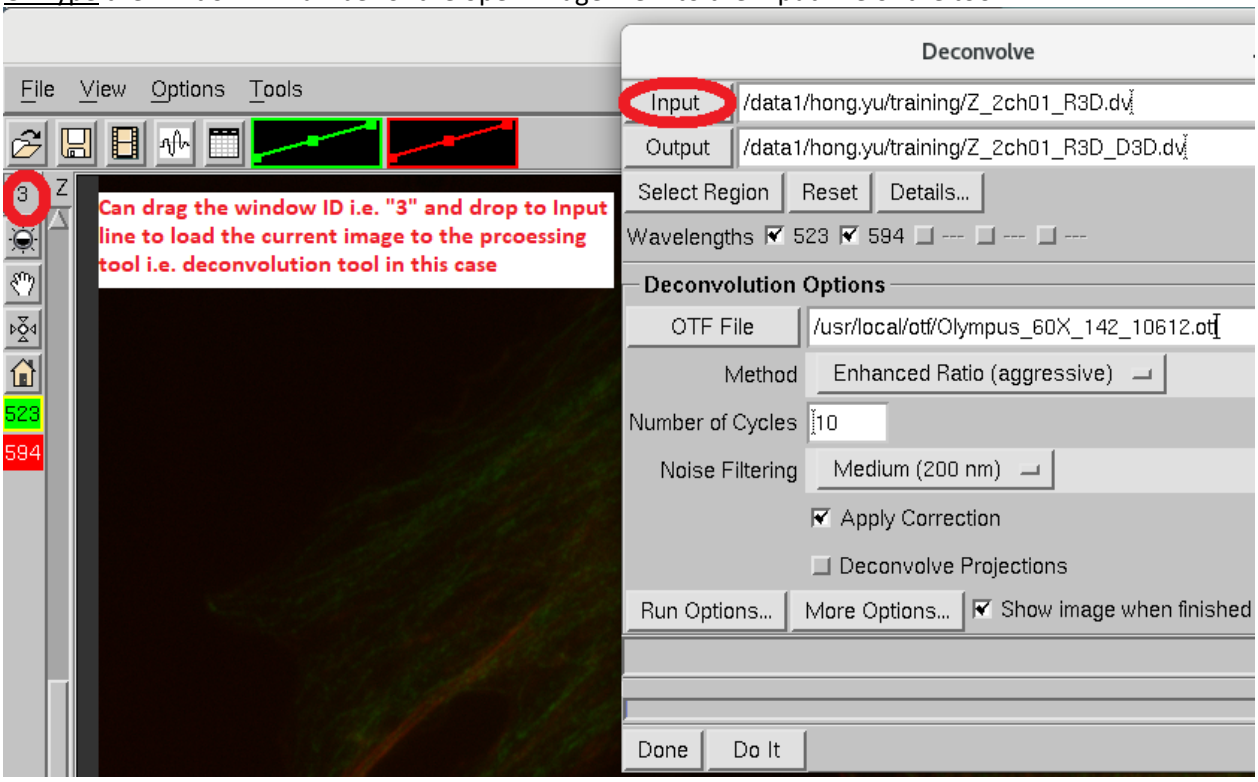
2.7.2.4 Save as Movie

In the image window, select “File”/”save as movie”. Select the preferred file format i.e. MPEG, AVI or Quicktime, and Frame Rate then select “Preview”. When the speed of the file looks ok, click “Ok” to confirm.

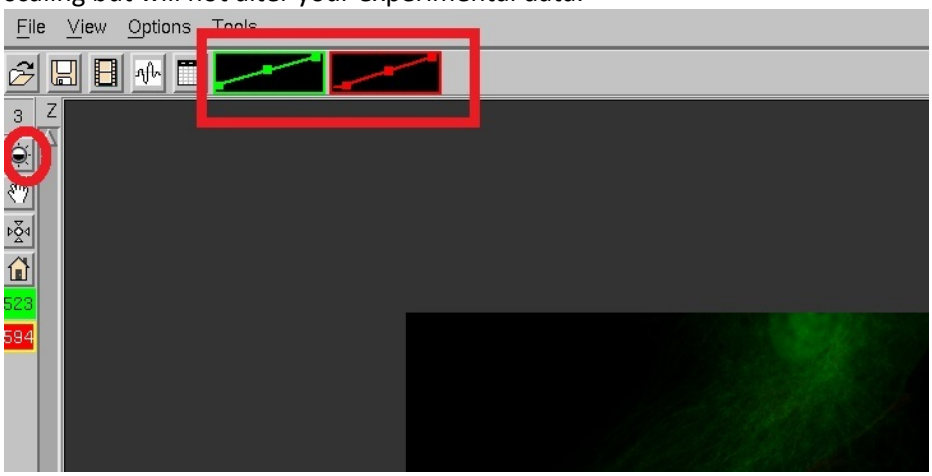
2.7.3 Image processing

2.7.3.1 General tips

- Loading files into processing tools i.e. deconvolution. First, open the tool, then you can either:
  Drag and drop the window ID number of the file into the Input line of the tool
  Or Drag and drop the image file icon from the data folder into the Input Line of the tool
  Or Type the window ID number of the open image file into the Input line of the tool

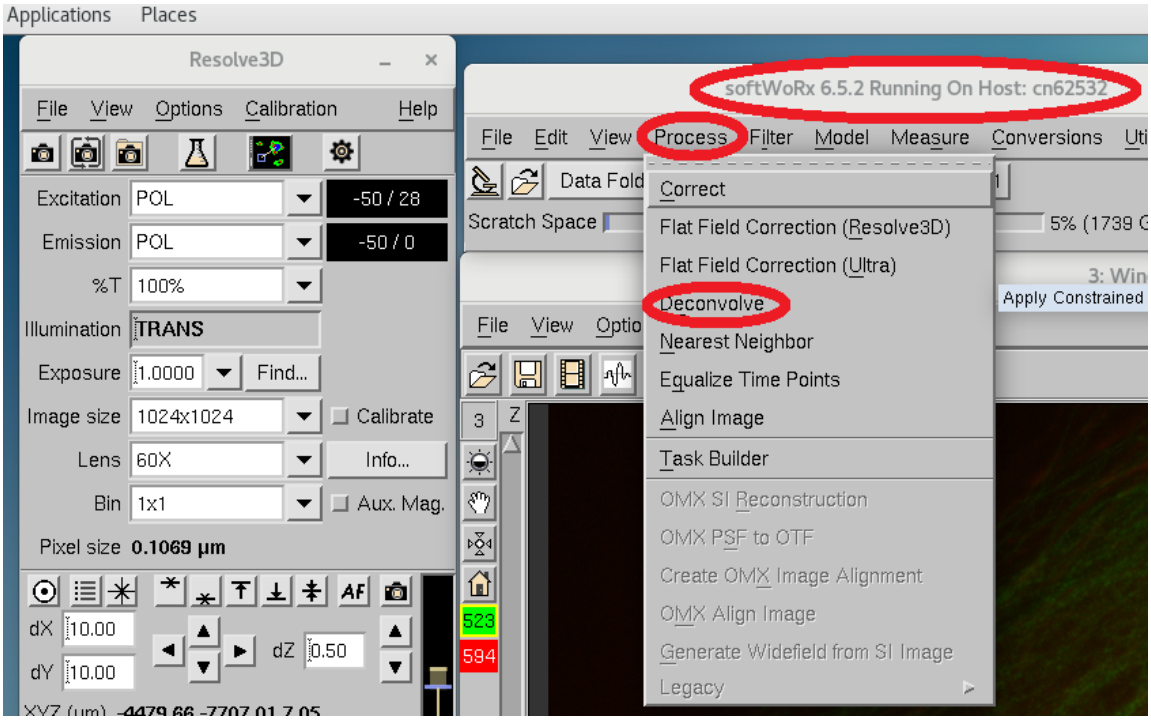


- Not all tools automatically save their output. You will be prompted to save unsaved files before closing.
- Histogram. Select the histogram button (red circle in the below screenshot) or use the small histograms located on the top of the image window (square in the below screenshot). This will adjust display scaling but will not alter your experimental data.



2.7.3.2 Deconvolution

- Open the Deconvolution tool in Softworx Section in the acquisition software: "Process"/"Deconvolution"



- Load the image to the Deconvolution tool using one of the methods described in Section 2.7.3.1. The information of the image will be loaded to the tool from the raw image file. Default settings are usually adequate for most applications.
- Process deconvolution. Click "Do it" to run deconvolution.

2.7.3.3 Quick projection

Open the Projection tool in Softworx Section in the acquisition software: "View"/"Quick projection". This will project your 3D data into 2D format by either Max intensity, Average or Sum method.

2.7.3.4 Volume Viewer

Open the Volume viewer tool in Softworx Section in the acquisition software: "View"/"Volume viewer".

DATA & RECORDS MANAGEMENT

All data must be saved onto the local computer (kept for 7 days before auto deleting): Data1\"firstname.surname". Your data gets transferred to the server automatically at mid night every day: Scientific Platforms (\\Scientific_Platforms\Cell Imaging\Deltavision\"firstname.surname" (kept for 14 days before auto deleting). NO USBs are allowed on the acquisition computer to minimise virus risk.

REFERENCES

The hardcopy of the full manual provided by the instrument vendor is available on the shelve above the microscope and on the Westmead Imaging site

DOCUMENT CONTROL

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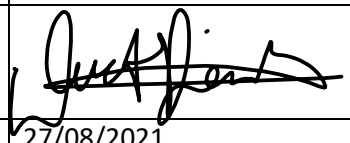
Signature of Approval Authority:	
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REVISION HISTORY

Revision Date	Version No.	Amended by:	Change