ELYRA P.S.1. PALM/STORM Guide

Getting Started

1. **In order to maintain mechanical stability the Elyra microscope should be left powered on with the incubator set to 30C. If you need to change the temperature you should notify other users by using the 'Incubator Warming up/Cooling down' check box on the PPMS booking page.**
2. If you need to restart the microscope for any reason, switch on the **3 main power boards from left to right** (two are located to the left of the microscope, one near the monitor)
3. Switch on X-cite HXP Lamp.
4. Switch on the computer and log in to PPMS.

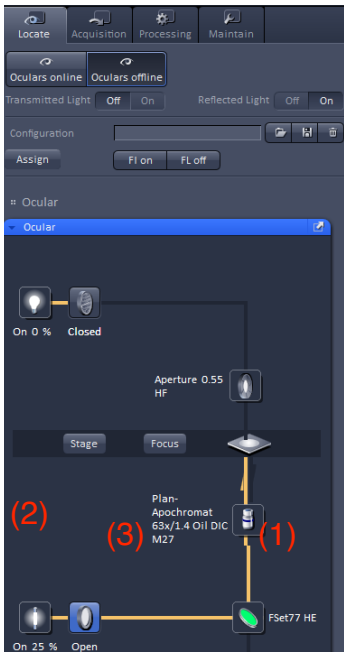
5. Start the **Zen 2012 Black** software. On the start-up window select "**Start System**".

Finishing your session

1. **Do not power down the microscope!**
2. Lower the stage and remove your sample and *gently wipe* any oil objectives you have used with *lens tissue* (**do not use kim wipes to clean objectives**).
3. Turn off the lasers within Zen.
4. Exit the software and move your files to your *home or group network/ USB drive/OMERO.*
5. Shut down the computer.

Visualising a Sample Through the Oculars

1. Use the **Locate** tab in Zen to visualise the sample.
2. Press the **Oculars Online** button to switch the microscope light-path 100% eyes.
3. Use the **Ocular** tool to configure the light-path
    a. Choose filter set 77 for visualisation (this is green/red dual filter) (1)
    b. Turn the X-Cite lamp to 'On' and **Open** shutter (2,3)



Configuring tracks

The Elyra PALM microscope is in essence a wide-field total internal reflection fluorescence (TIRF) microscope. It is equipped with 4 laser lines (405nm, 488nm, 561nm, 642nm) and has two reflector turrets depending on whether you need to perform single- or multi-channel experiments (see a Microscopy staff member for details).

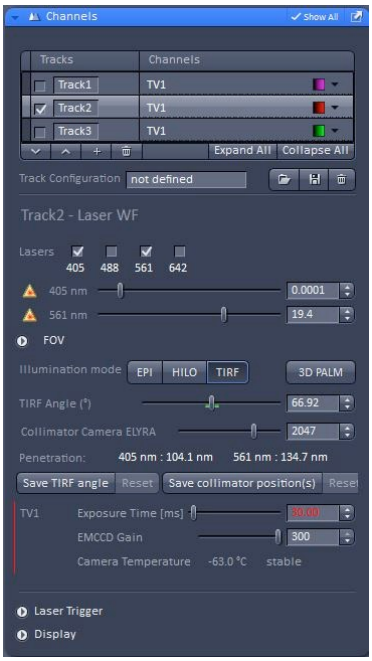
Tracks are easily configured as follows:

1. Turn on the required laser lines.
2. In the **Light Path** tool choose the **Laser WF** tab. The default camera for this acquisition mode is **TV 1** which is the EMCCD camera.
3. Select the laser line from 405nm, 488nm, 561nm and 642nm. (1)
4. Choose a reflector appropriate for the laser line and fluorophore being imaged. (2)
5. Choose an objective lens; the recommended for TIRF is **100x/1.46 Oil** lens. (3)
6. Use **Optvar** lens **1.6x** giving a pixel size of 100nm. (4)

Capturing time series data

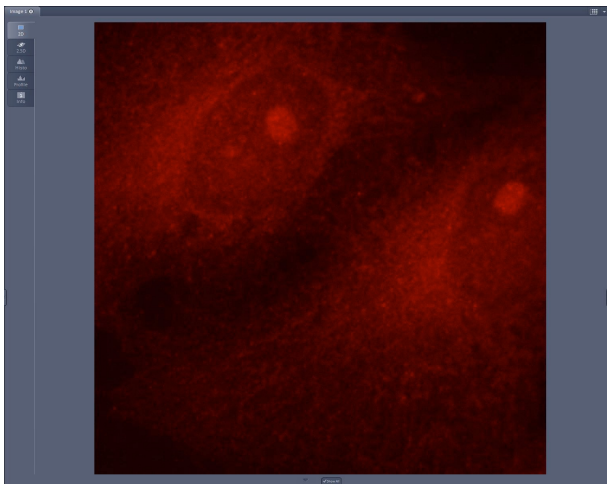
PALM/STORM is a pointillist technique where small populations of individual fluorophores are captured in a single frame and the acquisition of many thousands of frames allows the reconstruction of a high resolution image. As such it is necessary to capture of the order of 50,000 frames in a time series where the interval between frames is as small as possible.

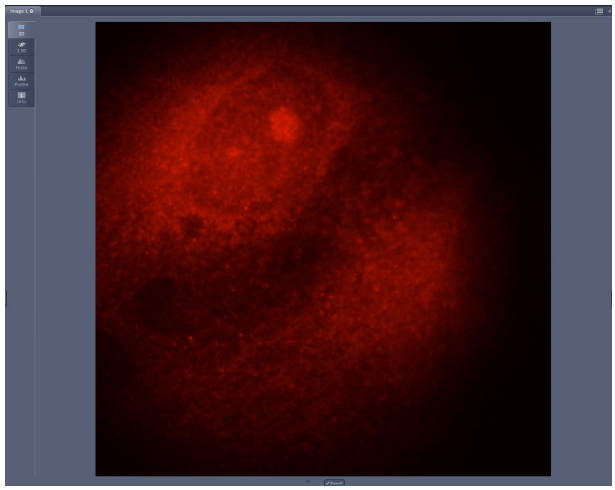
1. Use **Live** or **Continuous** acquisition along with **Channels** to set a laser power, TIRF angle and camera exposure time for each track.
2. Set the EMCCD gain for each track. A typical value is 300. The EMCCD gain has arbitrary units and a maximum value of 1000. As the gain increases beyond 300 an increasing proportion of readout noise is added to the image resulting in a signal-to-noise ration that reaches a plateau above 300.
3. The exposure time will be displayed in red when a value has been set that is incompatible with the frame size. This is due to the requirement that we readout frames as fast as possible from the camera - certain combinations of frame size and exposure time do not give optimal speed performance (e.g. for a frame size of 512x512 the exposure time should be > 30ms).



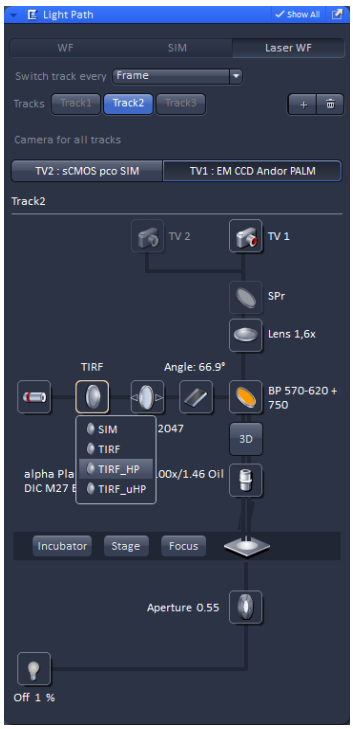
High power TIRF

PALM and STORM acquisitions can require high energy density at the sample plane in order to achieve the correct blinking rates. Use the TIRF collimator to reduce the field-of-view (FOV) being illuminated and concentrate the input laser power into a smaller area as shown below:



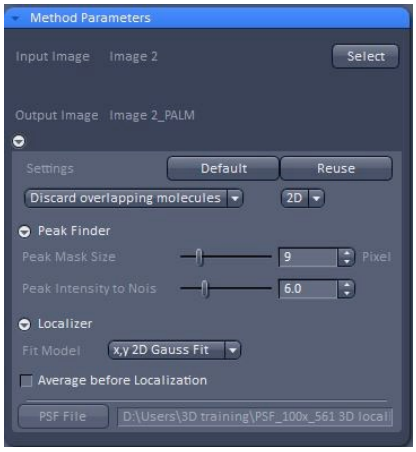


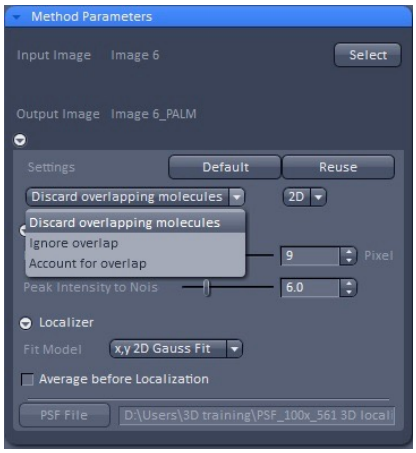
1. From the **TIRF** drop-down menu on the **Light Path** select **TIRF_HP**. **Do not use TIRF_uHP** - this is unsupported and the FOV will not be evenly illuminated.
2. TIRF angle adjustments can be made as described previously.



Processing PALM/STORM data

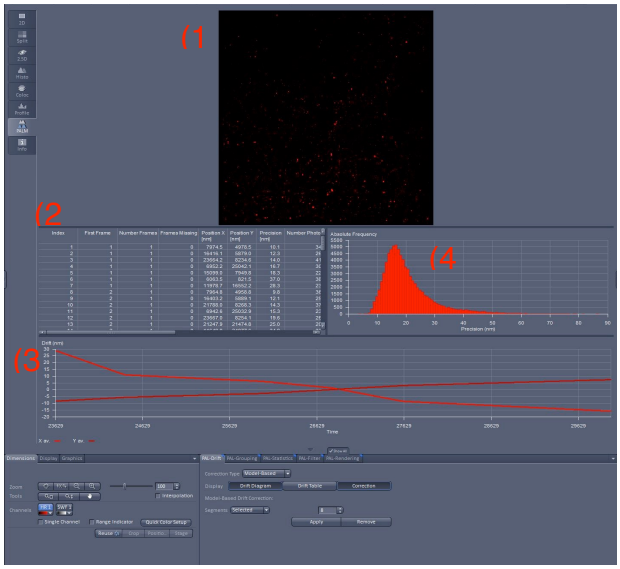
Zen Black integrates localisation microscopy image processing so that data can be acquired and immediately processed. Open source alternatives would be RapidSTORM, QuickPALM and Localizer.





1. Select the **Processing** tab.
2. Select **PALM** and then **PALM** from the resultant list.
3. Press the **Select** to choose the currently displayed image for processing.
4. Under **Settings** choose how to deal with overlapping fluorophores. Here the overlap is determined by the size of the mask using in **Peak Finder**. The following options are available:
   i. **Discard overlapping molecules** will remove any overlapping molecules from the analysis.
   ii. **Ignore overlap** retains the overlapping molecules in the analysis and ignores the fact that they overlap. This will increase the number of analysed molecules to the detriment of localisation precision.
   iii. **Account for overlap** uses a multi-object fitting routine to properly deal with overlapping objects. This is the slowest but most accurate way to process the data.
5. Use the **Peak Mask Size** to tune the amount of overlap between molecules in **Peak Finder**. This parameter will affect the way that the **Fit Model** is able to determine the molecule position. If the mask is too small the model will not be able to accurately determine the baseline and if it is too big there will be many overlapping molecules that will compromise the fit.
6. Adjust the Peak Intensity to Noise parameter to accept or reject dimly emitting fluorophores.
7. Under Localizer choose the Fit Model required. The default and best option is **x,y 2D Gauss Fit**.
8. Do not choose **Average before Localization**.
9. Press **Apply** to run the processing.

Post-processing PALM/STORM data

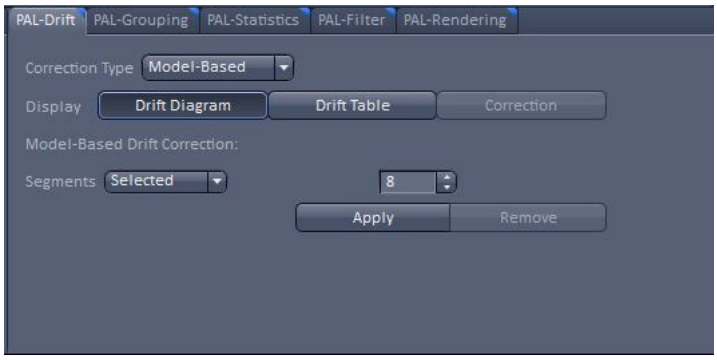


After processing a new PALM image is generated by Zen. This image contains the reconstructed super resolution image (1) and the data table from which the reconstructed image is derived (2), drift correction data (3) as well as a histogram plot of any the parameters determined during the processing procedure (4). The data tables can be saved by clicking the right mouse button and the PALM itself can be saved using **Convert To Image**.

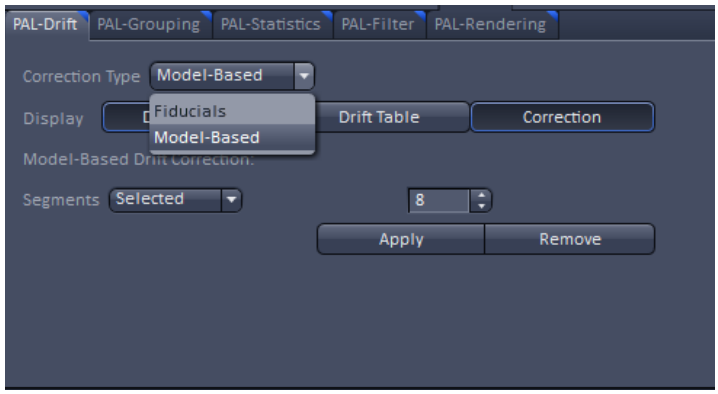
The bottom center panel contains a number of tabs for post processing PALM/STORM data:

1. PAL-Drift

This options allows the effects of mechanical drift in xy to be corrected. This can be done using either **Fiducials** or **Model-Based** corrections. Fiducials refers to the use of fluorescent beads or gold nano-particles placed as markers in the sample which are bright and do not blink allowing their location to be accurately tracked throughout the duration of the time series recording.

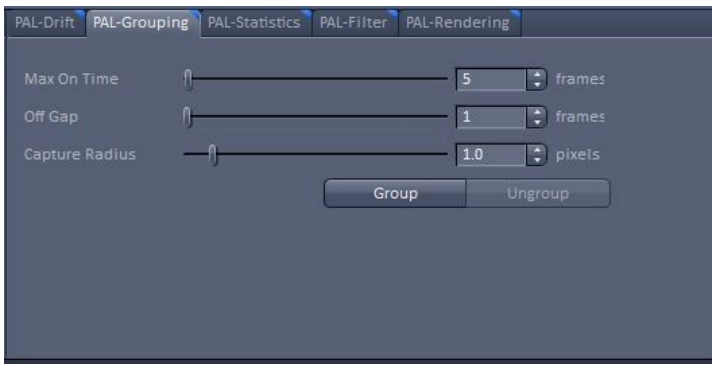


Model-Based correction is a mathematical to detect sample drift. Here the data-set is split into time **Segments**, the number of which is selected by the user. Each segment is cross-correlated and a drift diagram is generated as in (3) above. The user should increase the number of segments until the number of discontinuities in the the x- and y-drift stops increasing.



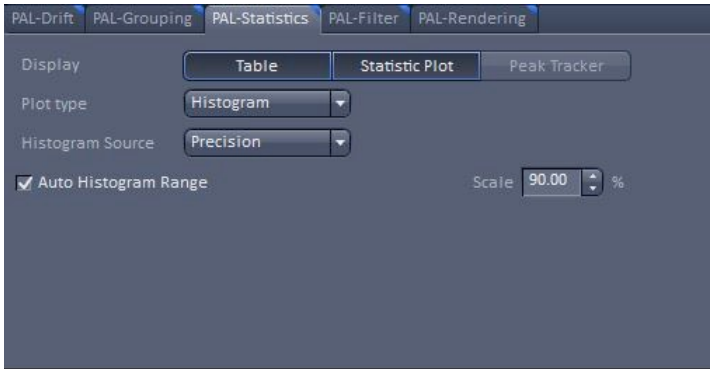
2. PAL-Grouping

The premise of both PALM and STORM experiments is that fluorophores stochastically switch on frame-to-frame such that the entire structure is sampled. However, each fluorophore can remain emitting for more than a single frame meaning that it can be detected and retained in the dataset for several sequential frames. This is to the detriment of the final image resolution since these sequential detections are treated as if they are unique fluorophores. Grouping seeks to address this by tracking each detected fluorophore to determine if appeared in several sequential frames. Set the **Max On Time** to determine how many sequential frames we expect a fluorophore to be emitting. The **Off Gap** parameter allows a fluorophore to stop emitting for the set amount frames and still considered to be the same object. The **Capture Radius** parameter tries to group only objects in sequential frames that are within this radius. Press **Group** to produce an average position and amplitude for objects meeting these criteria.



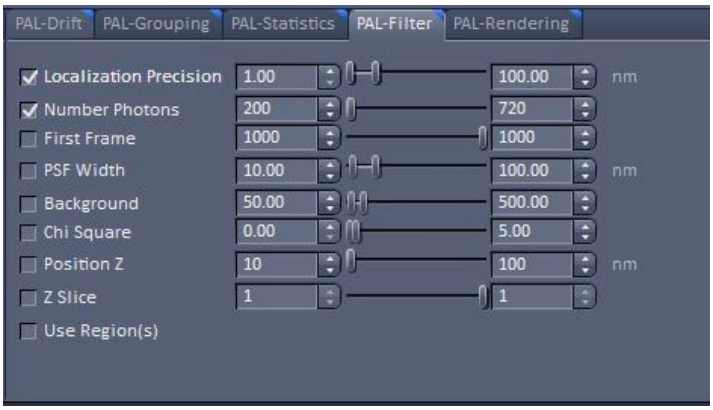
3.PAL-Statistics

This tab allows the user to decide on what parameter gets plotted in the graph displayed in (4). This can either be a histogram or a scatterplot and the source of the data can be any parameter listed in table (2).



4.PAL-Filter

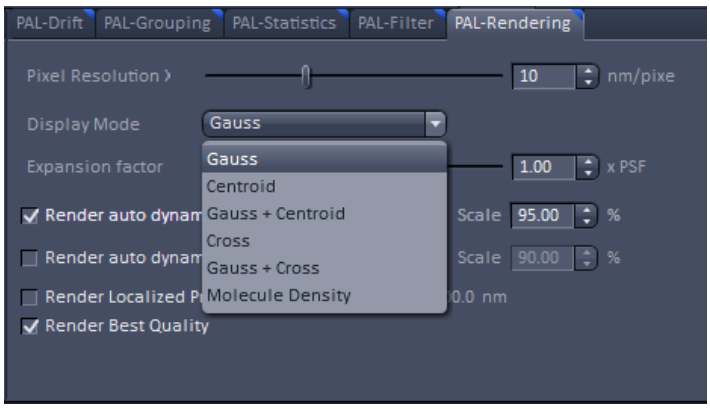
Since the super resolved PALM image is constructed from the list of localisations we can potentially improve the appearance of the image by filtering the localisations based on any of the parameters listed in table (2). The most obvious choice is the localisation precision. By default we filter out any localisations where the precision was above 100nm but improvements can be made by tighten the range (often spurious, or background events will have a large value for precision).



5.PAL-Rendering

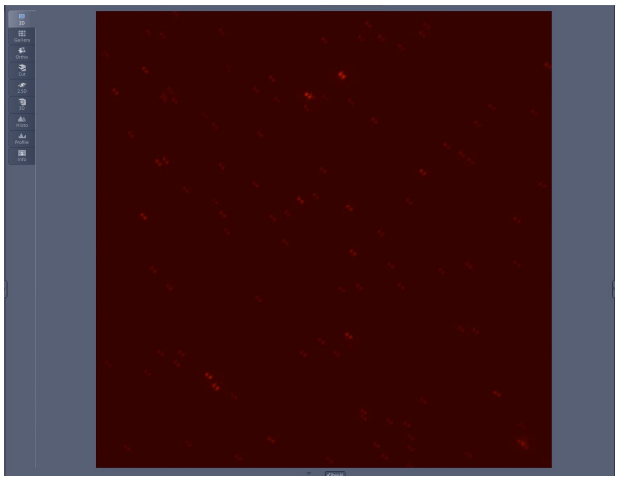
The PAL-Rendering tab determines the appearance of the final reconstructed image.

1. Set the pixel resolution. The value chosen here is typically no less that half the mean value of the localisation precision achieved (see (4) above).
2. Choose the **Display Mode**.
   i. **Gauss** will render each localisation as a 2D Gaussian with a standard deviation determined from its precision.
   ii. **Centroid** plots a marker at the arithmetic mean position of the each localisation.
   iii. **Gauss + Centroid** plots boths (i) and (ii) above.
   iv. **Cross** marks the (x,y) position of each localisation as a cross.
   v. **Gauss + Cross** plots both (i) and (iv) above.
   vi. **Molecule Density** constructs a 2D histogram from the all localisations.

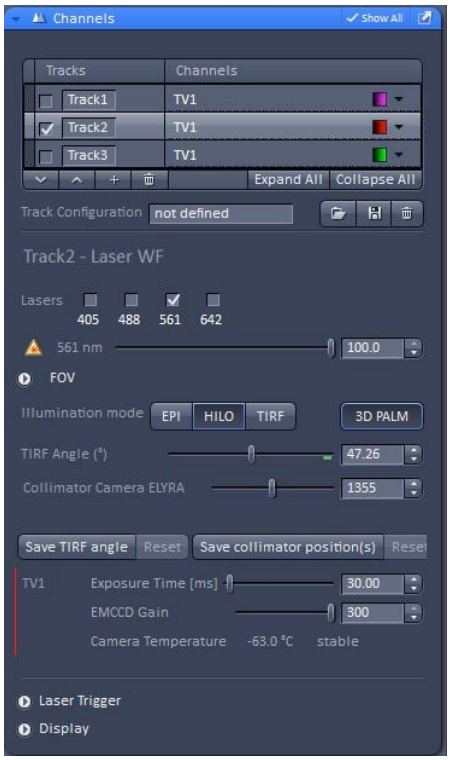


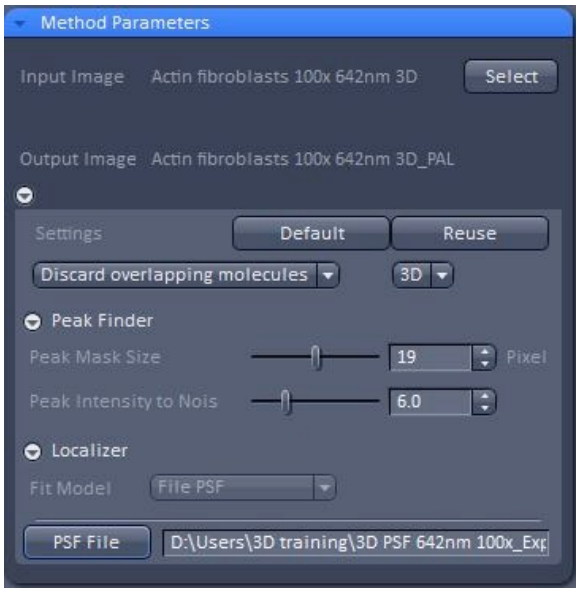
3D PALM/STORM imaging

3D imaging is achieved using an insert that produces a double-helix PSF in the image plane. As the z-position changes so does the rotation of the split PSF in the image. Tracking this rotation is used to produce a total z range of 4um.



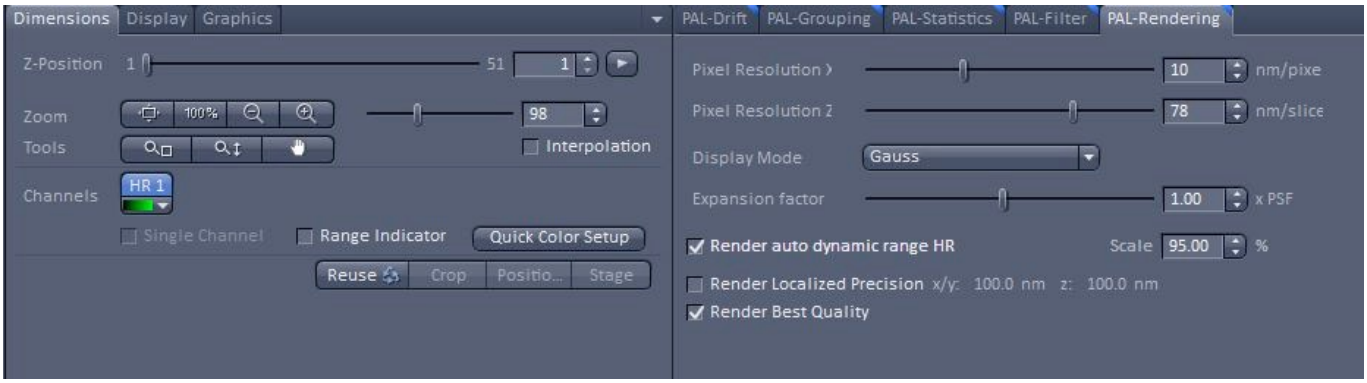
1. To activate 3D imaging press the 3D PALM button either on the **Light Path** tool or the **Channels** tool.
2. Capture data using either a time series, z stack or both. The critical thing to remember is that each z-plane with contain 4um of z information.
3. To process the data choose **PALM** and the **PALM** in the drop down menu from the Processing tab.
4. Press **Select** to process the image currently being displayed.
5. Zen will detect that this is a 3D data set. Choose how to deal with overlapping emitters (see "Processing PALM/ STORM data" for details).
6. Under Peak Finder set Peak Mask Size. Here the mask size is bigger than in the case of a 2D data set due to the increased size of the PSF.
7. Select a PSF File. This must be a 'localisation precision' dataset generated from sub-resolution fluorescent beads. The PSF File datasets can be found in "C:\Users\QBI Microscopy\Desktop\3D PALM PSF" and are named to indicate the wavelength of fluorescent bead that was imaged.
8. Press **Apply** to process the dataset.





Post-processing 3D PALM/STORM data

The new PALM image produced in Zen will initially be a 2D dataset until the **Pixel Resolution Z** is set. Initially this parameter is set to the total z range available which is 4000nm (4um).



1. Go to the PAL-Rendering tab and use either the slider or spin-box to set Pixel Resolution Z to a value other than 4000nm. This value chosen should be guided by the localisation precision achieved (see PAL-Statistics). Set a value no less that half of the mean value of the z localisaition precision. Note that generally the localisation precision in x and y will be worse in a 3D dataset than in a 2D dataset.

WORKING WITH CONFOCAL AND TIRF MICROSCOPES

Ergonomics: Use of mouse and keyboard / viewing computer screen – Prolonged use of the microscope and microscope computer without breaks can increase the risk of muscular strain.

Eye strain and fatigue – Viewing samples through microscope eye piece or computer monitor over lengthy periods of time can result in eyestrain and headaches.

Exposure to sharps – Exposure to razor blades, scalpels, forceps, cover slips, glass slides could result in cuts or puncture wounds to hands or other areas of the body. Any microscope slide shards or glass debris must be disposed of in the appropriate shapes disposable bin in accordance with PC2 regulations.

Exposure to intense fluorescent and laser light – Lasers and a xenon light source are attached to this microscope and are the source of intense and potentially dangerous light. Under no circumstances should any optical elements be removed from the microscope light path or fail-safe switches be circumvented. Do not attempt to adjust the lasers, laser light path, or laser modules in any way. Avoid direct exposure to the light.

Scope

This procedure details the method for using the microscopes equipped with laser light sources.

Safety Considerations

Personal Protective Equipment (PPE):

Laboratory coat, **latex gloves** and **closed in shoes** should be worn to prevent injury.

Ergonomics and Risk Exposure:

Appropriate ergonomics, including adjustment of the seat, computer screen and microscope oculars should be undertaken to reduce risk of strain injuries.

Emergency Procedures:

First aid may be required for:

Exposure to sharps – Contact the nearest first aid officer from the list that is beside all first aid kits and on safety notice board.

Exposure to intense fluorescent and laser light – Seek immediate medical assistance if you have been exposed to intense direct light or laser light.

In the event of a laser accident, do the following:

1. **Shut down the laser system.**
2. **Provide for the safety of personnel (first aid, evacuation, etc). If needed, provide further medical assistance for Eye Injuries by:**
   Proceed directly to: **Royal Brisbane and Women's Hospital at**
   Cnr Butterfield St and Bowen Bridge Rd
   HERSTON, QUEENSLAND AUSTRALIA 4029
   (07) 3636 8222

   Note: If a laser eye injury is suspected, have the injured person keep still and looking straight up to restrict bleeding in the eye. Laser eye injuries should be evaluated by a physician as soon as possible.
3. **Contact UQ Security Emergency on 336 5333.**
4. **Inform QBI's Laser Safety Officer, Rumelo Amor on 04 4907 8485, of the accident as soon as possible.**
5. **A UQ online incident report must be completed as soon as possible after the incident.**

All incidents must be reported to the OH&S Manager and on UQs online incident reporting system.

Contacts: Security x53333 or OH&S Manager Ross Dixon 0401 673 654