2D PALM/STORM (Elyra Super Resolution)

Application	Sample Thickness	Objective	Camera
SIM	< 20 um	63x/1.4 oil	sCMOS TV2
PALM/STORM/TIRF	< 4 um	100x/1.46 oil	EMCCD TV1

Track Configuration

1. Turn on the required laser lines.

2. Choose the 100x/1.46 oil lens (1).

3. Select *Laser WF* mode to acquire PALM/STORM raw data images.

4. The 'TV1: EM CCD Andor PALM' button is automatically activated.

5. Setup light path:

   - Activate lasers required (2)

   - Use TIRF for FOV

   - Choose appropriate filters (3)

   - Set Optovar lens to 1.6 (4)

6. Select the appropriate frame size in 'Acquisition Mode'.

Light Path configuration for PALM/STORM/TIRF

Image Acquisition

1. Start with the 642 nm laser line (or line that excites your sample). In the *Channel* tool, start focusing sample on *Epi* illumination mode with a low laser power (up to 5%), high EMCCD gain (200) and 100 ms Exposure time.

2. In Light Path or under FOV, switch to the ultrahigh power field (*uHP*) to increase power density.

   TIRF-uHP mode gives ~ 1.5 kW/cm2 at sample for 642nm on 100x.

3. Switch to *TIRF* illumination mode. Adjust TIRF angle to get an image with the least background.

4. On the touch screen display of the microscope`s stand, activate Definite Focus with continuous stabilization to counteract any z-drift:

   *Home > Settings > Components > Focus > Continuous Operatio*n

   Then, activate Definite Focus: Home > Microscope > XYZ > Definite Focus: 'ON'

Channels tool shows multi-track setup, laser power, illumination mode (in box: Epi, HILO, TIRF) & other acquisition parameters

5. Define a time series in the 'Time Series" tool for at least 20,000 frames. This frame number is mostly sufficient to bleach all available molecules.

6. Decrease exposure time to 30-50 ms (you may have to decrease the EMCCD gain first). Increase laser power stepwise to 100% to avoid overexposure of the camera.

7. Once the molecules are brought continuously to their dark states, increase EMCCD gain until you reach 200-300V without overexposure. You should now see individual molecules blinking. If molecules overlap heavily, wait with these settings until you can distinguish single molecules before recording.

   If your fluorophore does not blink, you may have to activate the 405 nm or the 488 nm laser line for activation. Use low laser power for the activation laser (e.g. 0.001 to 1%).

8. Start recording time series (Start Experiment).

Processing of 2D PALM/STORM Images

1. Select the Processing tab.

2. Select PALM and then PALM from the resultant list.

3. Press Select to choose the currently displayed image for processing.

4. Under Settings, choose how to deal with overlapping fluorophores. Overlap is determined by the size of the mask used in Peak Finder. The ff. options are available:

   Discard overlapping molecules will remove any overlapping molecules from the analysis.

   Ignore overlap retains the overlapping molecules in the analysis and ignores the fact that they overlap. This will increase the number of analyzed molecules to the detriment of localization precision.

   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.


Image processing: Overlap options

5. Use Peak Mask Size to tune amount of overlap between molecules in *Peak Finder*. This will affect the way the Fit Model determines molecule position. If mask is too small, the model will not accurately determine the baseline, and if 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 Fit Model. Best option: x,y 2D Gauss Fit.

Image processing: Peak Finder and Localizer (fit model)

8. Do not choose Average before Localization.

9. Press Apply to run the processing.

Post-Processing of 2D PALM/STORM Images

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.
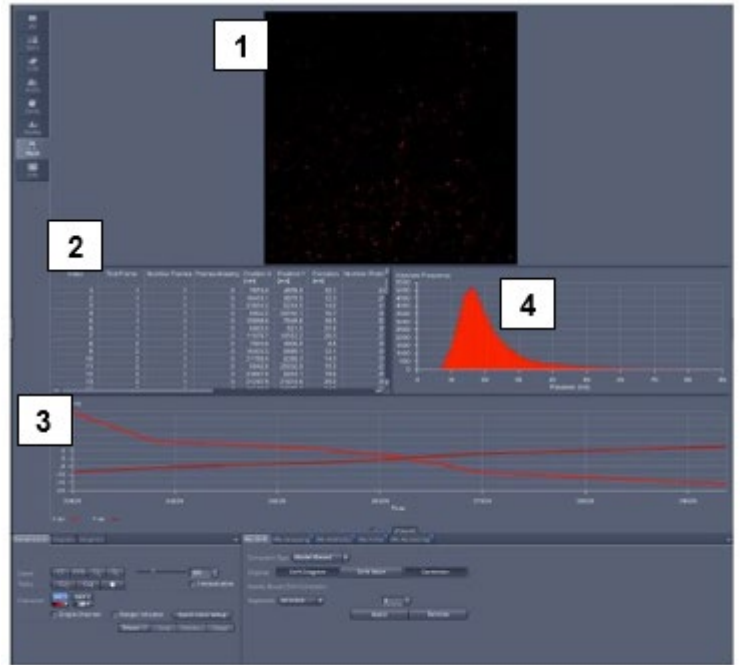
Window showing reconstructed image (1), data table (2), drift correction plot (3), and histogram plot (4)

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

1. PAL-Drift

This option 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 nanoparticles 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 is a mathematical correction to detect sample drift. Here the dataset is split into time Segments, the number of which is selected by the user. Each segment is cross-correlated and a drift diagram (3) is generated.
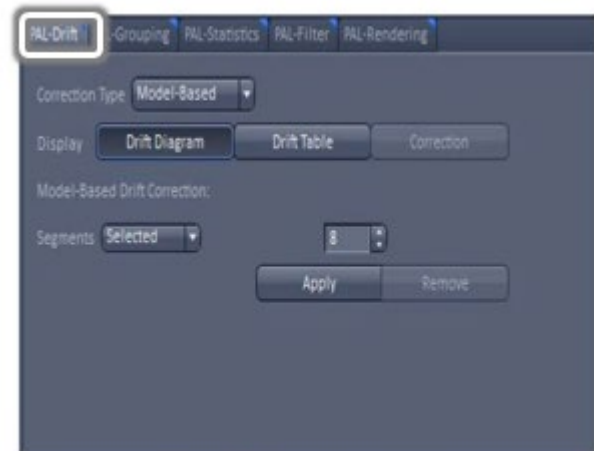
PAL-Drift option window

2. PAL-Grouping

Grouping tracks each detected fluorophore to determine if it appeared in several sequential frames.

Max On Time determines how many sequential frames we expect a fluorophore to be emitting.

Off Gap allows a fluorophore to stop emitting for the set amount of frames and still considered to be the same object.

Capture Radius tries to group objects in sequential frames that are within this radius. Press *Group* to produce an average position and amplitude for objects meeting these criteria.

PAL-Grouping option window

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).

PAL-Statistics options window

4. PAL-Filter

We can potentially improve the appearance of the image by filtering the localizations based on any of the parameters listed in the data table. The most obvious choice is the localization precision. Filter out any localizations where the precision is above 100nm but improvements can be made by tightening the range (often spurious, or background events will have a large value for precision).
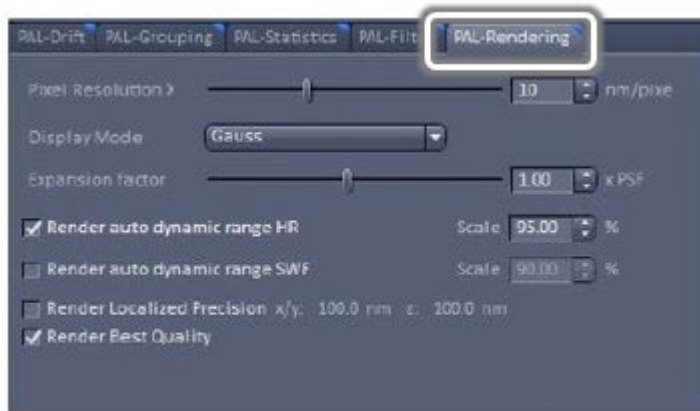

PAL-Filter option window

5. PAL-Rendering

This tab determines the appearance of the final reconstructed image.

- Set the pixel resolution (no less than half the mean value of the localization precision achieved in the PAL-Filter tab.

PAL Rendering option window

- Choose the Display Mode:

  a. *Gauss*: renders each localization as a 2D Gaussian with a standard deviation determined from its precision.

  b. *Centroid* plots a marker at the arithmetic mean position of the each localization.

  c. *Gauss + Centroid* plots both (a) and (b) above.

  d. *Cross* marks the (x,y) position of each localization as a cross.

  e. *Gauss + Cross* plots both (i) and (iv) above.

  f. *Molecule Density* constructs a 2D histogram from the all localizations.