

N-STORM System ESRIC@IGMM

To turn on system – see illustrated instructions on the notice board

The 647 laser is high power and does move in TIRF mode do not look down it directly and do not operate the system without prior training from ESRIC Staff

Please do not touch the 100x objective on this system. It has been calibrated for 3D N-STORM experiments



Danger: This system contains category 3B lasers and moving laser illumination.

Please report any faults with the equipment to local ESRIC staff (i.e. a person in the IGMM building)

Only trained users may use the system. Users are expected to comply with the SOP. Particularly parts regarding managing risk.

3D N-STORM manual

Single-molecule localization fluorescence microscopy (SMLM) consists in essence of creating a map of probable locations for individual fluorophores. After acquiring a sequence of images at a high frame-rate where fluorophores are cycled through one or multiple bright and dark states, the sequence is processed to produce a list of fluorophore positions and a probability density map for their distribution in the sample. The process from acquiring the image sequence to generating a quantifiable map can be split into three main steps – pre-processing, localization and rendering – that have to be done before analysing the super-resolved image and extracting quantifiable parameters.

Sample preparation recommendations:

The easiest STORM set up is single colour storm. Please use dyes containing AlexaFluor 647 or Cy5 as these blink well. Nanobodies (<http://www.chromotek.com/products/nano-boosters/>) or FaB fragments for secondary antibodies are recommended. These dyes will blink in aqueous Vectasheild and are good for STORM imaging for up to 2 weeks after mounting.

Samples can be prepared on the high precision coverslips (same as SIM, supplied in IGMM STORES) or glass bottomed dishes or Labtek 2 chambers.



Since most people can't visualise 647 by eye its recommended to include a fiducial marker which won't bleed through in to the 647 channel. 568 Phalloidin and 100nm Green/Blue microspheres have been successfully used. DAPI has a very long emission spectrum and mayn't be a good choice, however this varies from experiment to experiment.

Multicolour STORM is challenging. PALM and STORM can be combined which, providing a fluorescent protein is available works well. mEOS4 is a particularly good choice. 2 Colour storm is possible but buffers need to be used and the end user needs to optimise this themselves.

BEFORE Imaging is started:

The STORM drifts if it is not at constant temperature. To mitigate this heat the chamber to 28°C for 4 hours prior to experimentation and ensure the laser bed is switched on during this time. Ensure the STORM objective and any other equipment to be used is in the chamber equilibrating

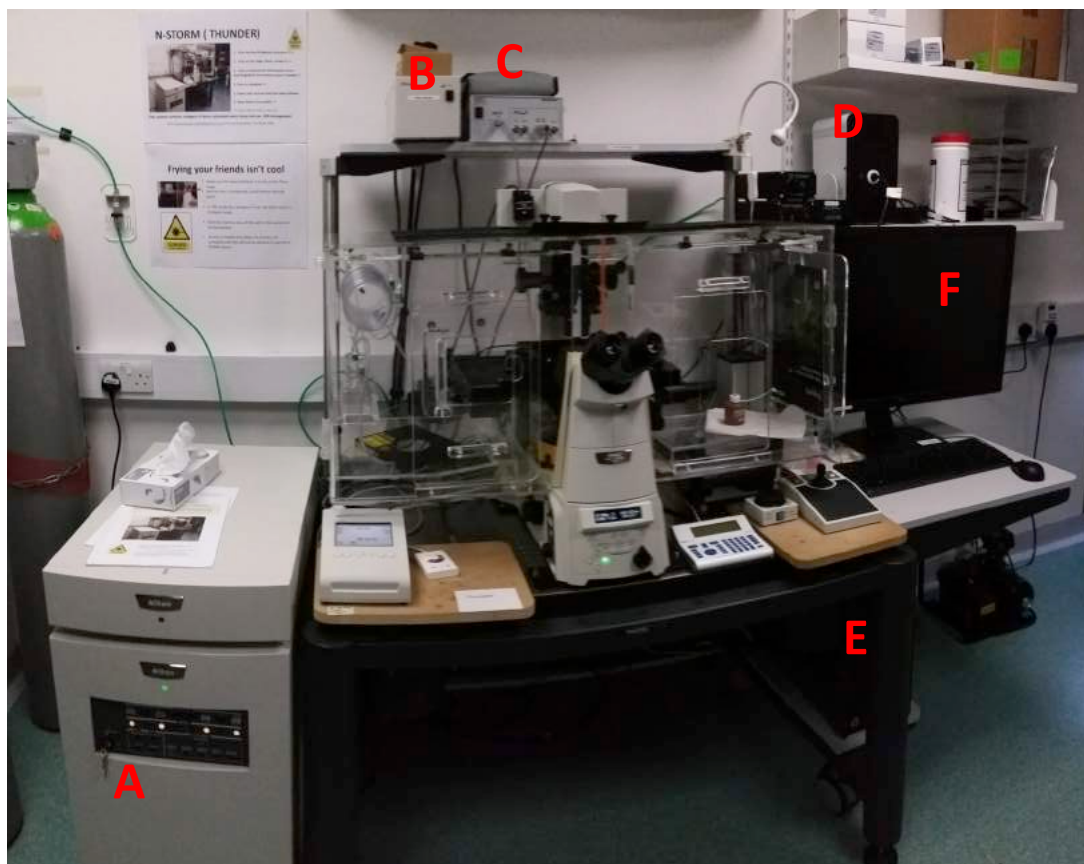
If carrying out **3D STORM** ensure the **Piezo is fitted onto the stage and is flat**. A facility member of staff may need to assist with this.

DURING Imaging

Try to keep the sample chambers temperature constant, use the small doors on the sample chamber to change and move samples to prevent the warm air escaping

Turning on the N-STORM system.

1. Turn on laser bed. Turn key 90 degrees to do this. If the individual laser wavelength buttons don't illuminate press them to turn them on
 - a. The lasers take about 5 – 10 minutes to warm up
2. Turn on the stage & Piezo, **B** and **C**
3. Turn on PE4000 LED Fluorescent Illumination source via the switch on the front **D**
4. Turn on computer **E**
5. Select user account and start Nikon software **F**
6. Open Storm-User profile



Laser Hazard

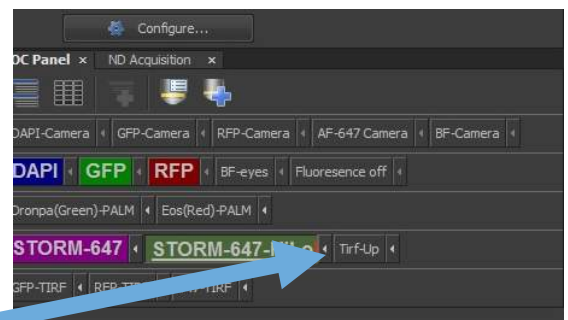
Align the TIRF illumination on the ceiling to ensure the beam is using Kohler illumination

You need to be shown by AIR staff (Ann or Matt) at time of writing how to Align the TIRF. Please be aware that the laser beam is category 3B. **Do not look directly into the TIRF beam**

There is a cross on the ceiling. The TIRF should be aligned to this. The head of the Microscope (TI) needs to be moved back so the illumination from the lens can be centred and focussed on the ceiling.

Ensure the 100x TIRF lens is selected

Bring the lens up to the focal plane, ideally you would know the z value to move to by focussing on a sample first. Then remove the sample and clean the oil off the lens.



To set up the illumination firstly select TIRF up from the OC panel.

Next set the laser power in the N-STORM panel to 0.2 – 2% and check the 647 laser is on

Press the shutter button to open the shutter

Push the microscope head back so the light focusses on the ceiling



Setting Kohler Illumination for TIRF

Generally the TIRF illumination will not be focussed in the centre of the spot

To do this, adjust the path of the TIRF and beam:

- Forwards and backwards using the Silver knob on the TIRF illuminator. (*)
- Left and right using the Automated TIRF Mirror in NIS elements
- The focus of the beam is adjusted using the small black knob on the TIRF illuminator (*)



Focussed TIRF



The black screw then focusses the TIRF in place

Once the illumination is focussed on the spot on the ceiling the TIRF is aligned.

- Turn off the shutter in the N-STORM Menu
- Select STORM 647 or STORM Hilo in the menu
- Using the VFL GUI turn up the 647 power to 300mw for STORM or 100mw for TIRF

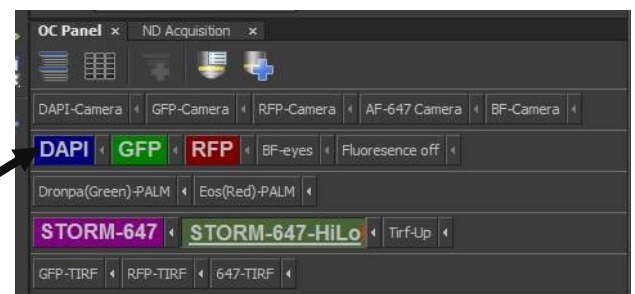


When the TIRF is focussed it will form a Gaussian distribution on the ceiling with. If it is poorly aligned the spot will be diffuse. If the spot is not completely circular there is a fault with the objective. The objective should be completely clean



Locate the sample (1 colour 647)

- To find the sample by eye select one of the Eyes Optical Configurations (OC's). There are now several distinct filter sets on the system for viewing by eye. Individual wavelength cubes for DAPI, FITC or TxRd. Alternatively there is now a multiband quad and triple set for the following wavelengths DAPI/GFP/TRITC/Cy5 and CFP/YFP/mCherry. The OC's are labelled as such.

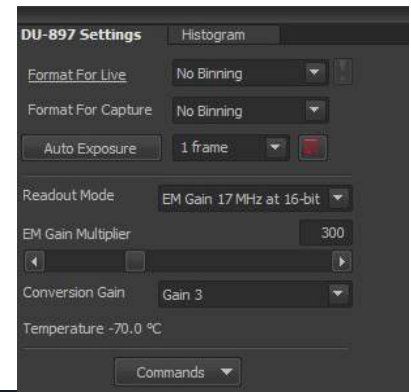


- Ensure the sample is in the very centre of the field of view. If included use the fiducial marker to locate the sample.

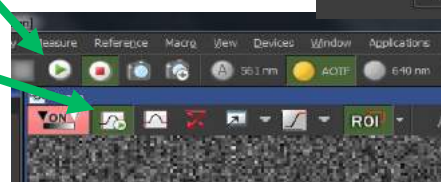
3. Switch on the Perfect focus system
 - a. To locate the sample on the camera select **STORM 647** which will go into TIRF illumination or **STORM-647 HiLo** which will use near TIRF.

4. Set up the camera, Ensure the setting are

- a. Readout mode: EM Gain 17MHz at 16 bit
- b. EM Gain Multiplier: 300
- c. Conversion Gain: 3
- d. Adjust autoexposure, generally 1 frame is ok.

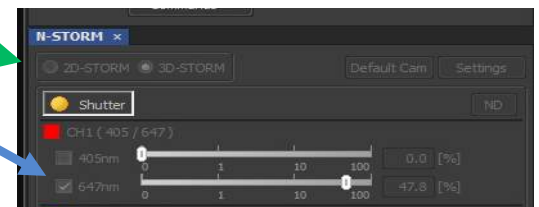


5. Visualise the sample on the camera
 - a. Press the Green Arrow in the top menu
 - b. Press autostretch (adjusts histogram)

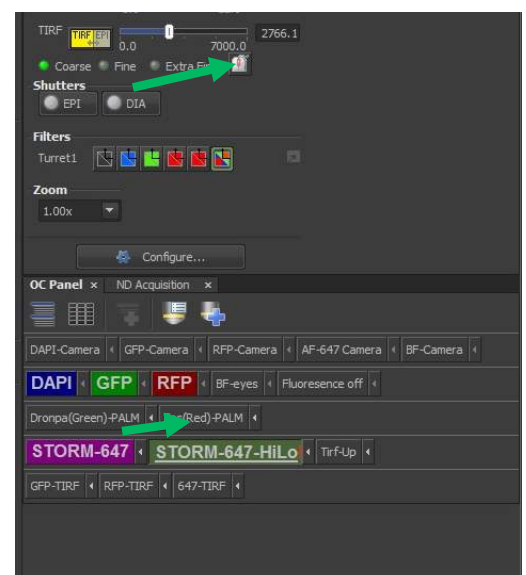


image

6. In the STORM GUI remove the **Interlock**
7. Open the shutter
8. Ensure the 647 laser box is checked
9. Turn up the 647 laser power to 5% or more
10. The sample should now be visible on the camera.
Focus axially (z) using the PFS controller.

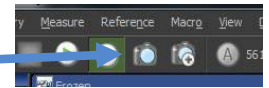


- The TIRF angle will also need to be focussed for your sample. This is done by adjusting the angle of the TIRF mirror in the Ti pad (LHS) controller. Clicking on the white square icon allows mouse wheel control of this.
- For TIRF the angle needs to be adjusted so the background is black and the sample is visible, this is true Total Internal reflection. The value for the angle should be around 2400
- For Hi Lo a higher value is needed in the TIRF angle controller, generally 2700. However there will be background present
- If you are carrying out 2 colour STORM or using EOS, Dronpa or other PSGFPs for PALM you will need to use the Multicolour STORM / TIRF or PALM settings.

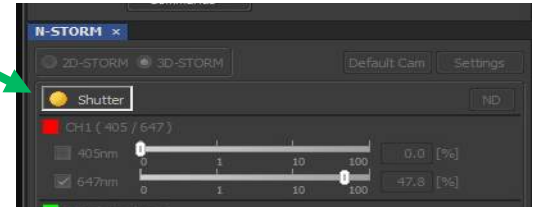


Set up STORM Imaging

To acquire an image before STORM commences press the camera icon in the top menu and save the image.



Switch off the shutter in the STORM GUI to turn off laser light. Press the red square button in the top menu to turn off the camera.



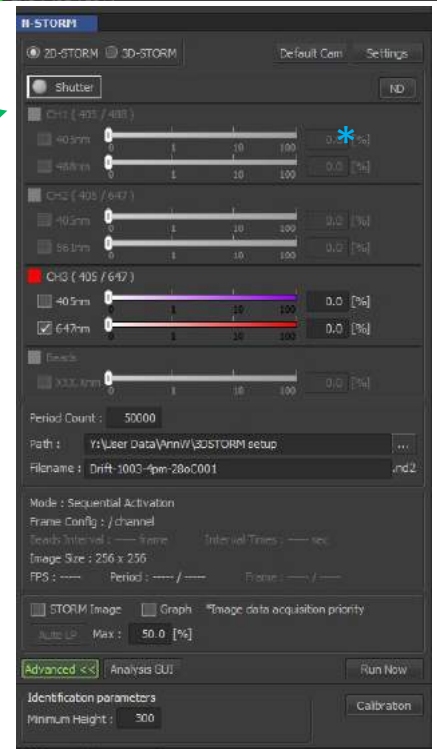
1) Start STORM Imaging GUI SetUP

First of all check the sort of STORM imaging to be carried out 2D or 3D, select the radio button as appropriate. If 3D ensure the astigmatic lens is in front of the camera.

IMPORTANT: Ensure the camera is set up as described above as the internal software will convert photons to electrons, but assumes the settings are as recommended here.

For all experiments its important to set up the:

- 'Period count' which is the number of images to be acquired in the STORM dataset
- The Path that the images are to be stored to
- The filename that the Data will have.



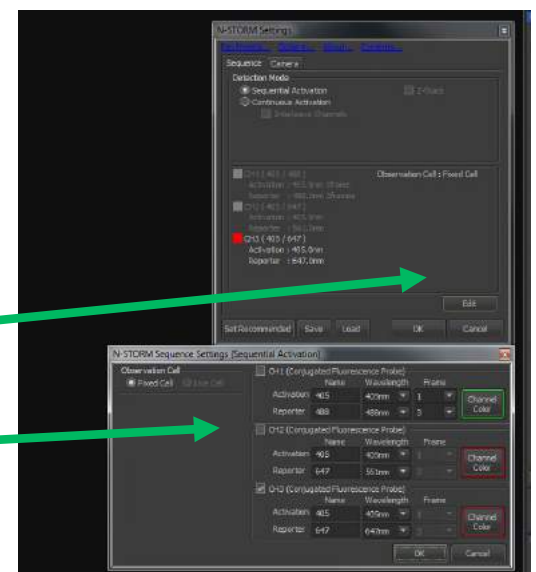
To control the way the STORM data are acquired Press Settings at the top of the STORM GUI (*)

The **N-STORM Settings** dialogue will pop out. Settings are as follows:

Sequential activation – Will add a pulse of activation light and a pulse of Reporter light and switch between channels on different frames. This effectively concatenates multicolour channels. Minimises drift between channels but photoswitches may be missed.

To set up how the channels are sequentially activated press edit. The Sequence Settings dialogue pops out

Here the activation and reporter lasers can be adjusted and multi-colour imaging set up. Ensure the reporter lasers match the fluorophores used.

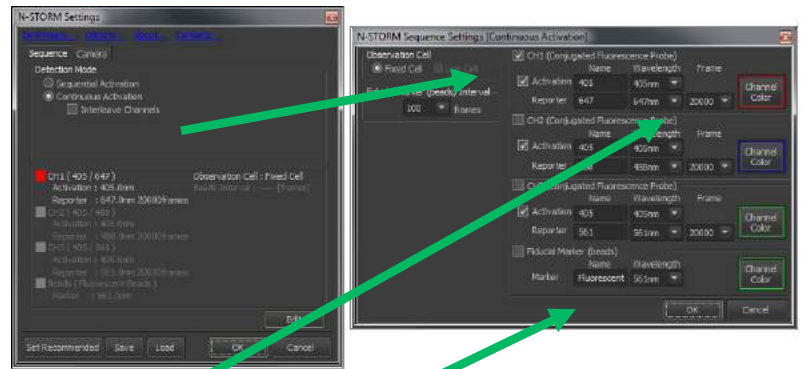


Continuous Activation: here the activation and reporter lasers are on permanently.

In the Sequence settings multi-colour imaging set up. Ensure the reporter lasers match the fluorophores used.

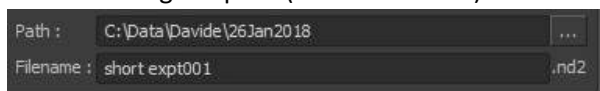
In standard continuous activation mode first one channel is acquired and then the second so there may be drift issues between channels. Set the number of images per channel

Drift correction can be added using fiducial markers to correct for drift. Select the colour of beads

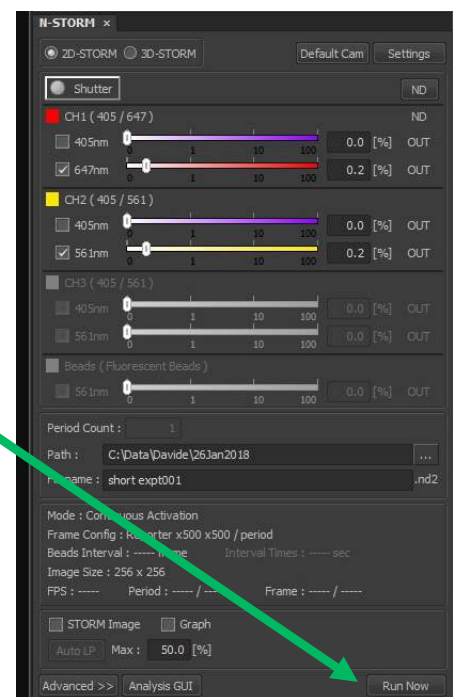


2) Run STORM experiment.

- Set the saving file path (data autosaves)

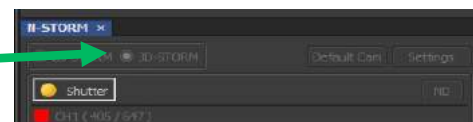


- With the camera active and shutter on
- Set the 647 laser to 100% power – The screen should go white and eventually blinking will be seen. Laser power can be reduced to ensure an appropriate amount of blinking is present. Too many / overlapping blinks are difficult to fit.
- For 2 colour the second laser can be added in as well.
- Once the frequency of blinking is acceptable Close the shutter And press press **Run Now**
- It is possible to have a 'on the fly' STORM image and graph shown however this means the live image runs more slowly



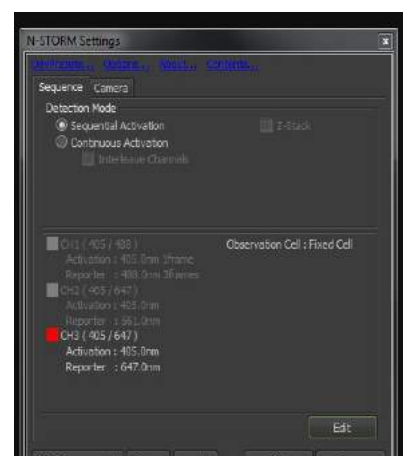
3) 3D STORM experiment – Requires Piezo Stage + Fiducial markers.

The Channel set up here is identical to a 2D experiment except 3D STORM should be checked in the N-STORM manual + the astigmatic lens placed in the light path.

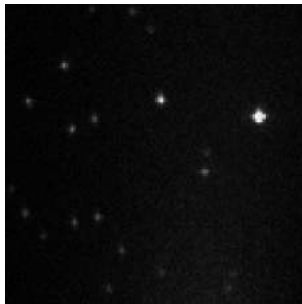


In the Settings dialogue there is the Z stack option available this enables Z stacking in 3D STORM which is useful.

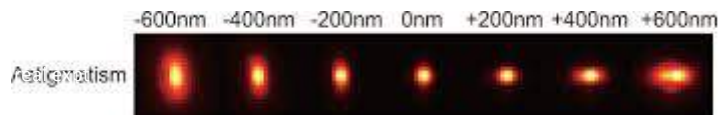
If doing Z stacking it is recommended to use 5000 frames per STORM experiment as each Z stack is treated by the software as a separate STORM experiment (See later). Please remember to set up channels accordingly too.



When doing 3D STORM the astigmatic lens needs to be in. It is also important to check on your sample that true astigmatism can be seen in your sample. Fiducial markers can help with this,



Fiducial marker – such as 100nm 488 beads will show a PSF stretch to correspond with the axial position.

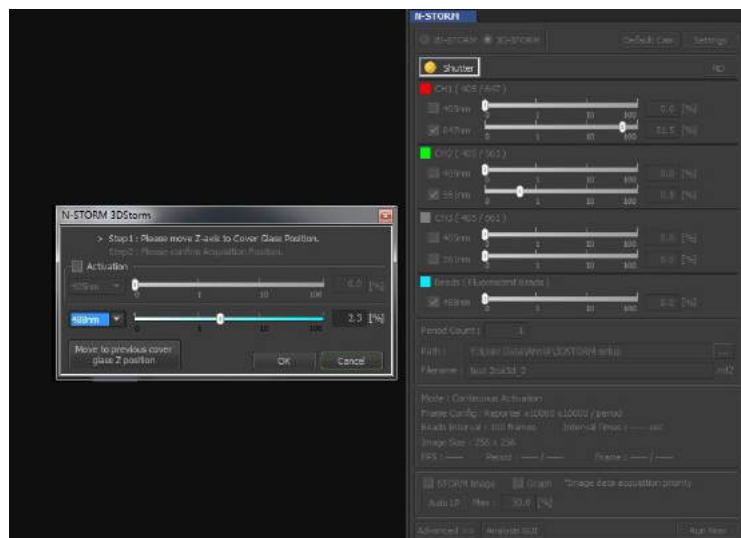


Once the data path has been set in the N-STORM dialogue, the shutter opened and the laser powers set (100%)

Press **Run Now** (+/- Z stacks.)

The 3D Storm 'axial drift alignment' dialogue will appear. This has two steps:

- 1) Using the fiducial markers set where the coverslip is. Be sure to select the correct laser and laser power. Press OK
- 2) Focus on the Z plane of the sample, for this select the imaging laser (usually 647) and laser power. Press OK

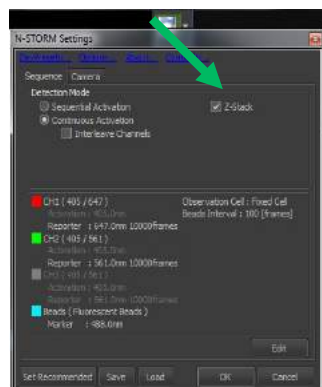


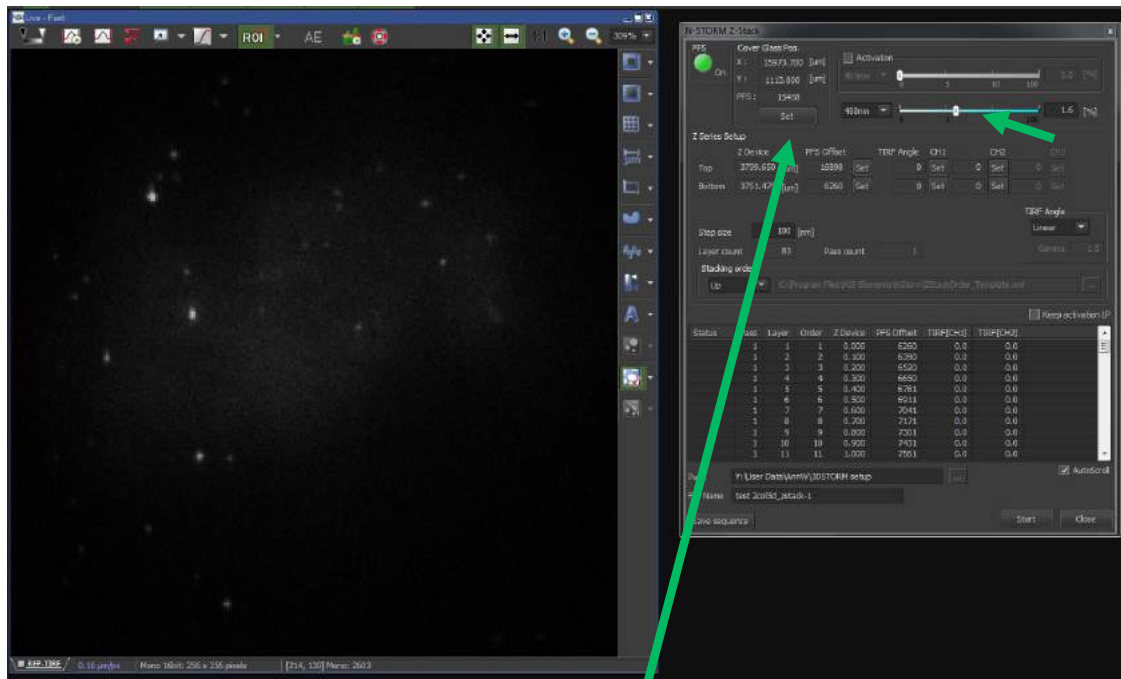
Setting up Z stacks for STORM

It is very important the system is totally stable and equilibrated at temperature for this otherwise the drift will make the data unusable

The acquisition parameters for the real experiment are set first (640nm laser at 100% etc). Once Run now is selected the Dialogue goes into a set up procedure for the Z stacks

If the Z stack option in the N-STORM settings has been checked).

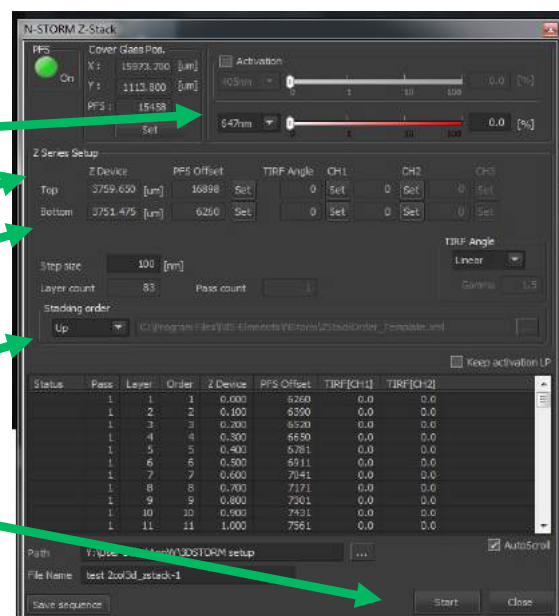




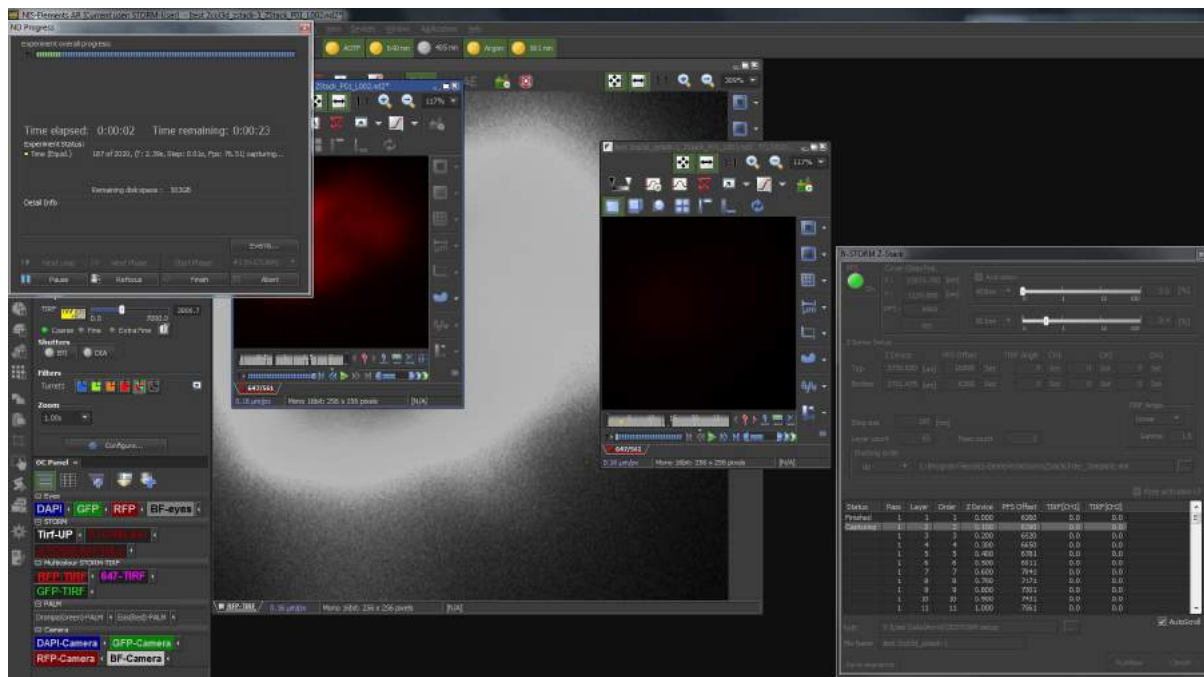
In this menu set the coverglass position first. Using fiducials and the appropriate laser. Press **SET**

Next, the Z series is set up.

- This is done using the channel of interest so the laser needs to be changed to the imaging laser at low power.
- The Axial and TIRF angles are both set and step size starting with the z position and TIRF angle at the top (both are set using separate SET buttons). Then the bottom z position and TIRF angle (again both set using appropriate SET buttons).
- The stacking order can be set and the TIRF angle. I used Linear. The Path and Filename of data are set. Then **START** is pressed.
- Acquisition of the data will now start.



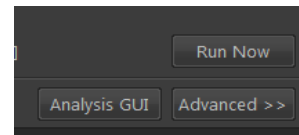
Each Z stack is cycled through and on the dialogue it is noted if the Z stack is capturing or finished.



N-STORM Data analysis:

The 2D or 3D settings that the images were acquired with will be included in the header file of the ND2. The Nikon analysis software will automatically 'know' whether a 2D or 3D experiment has been carried out.

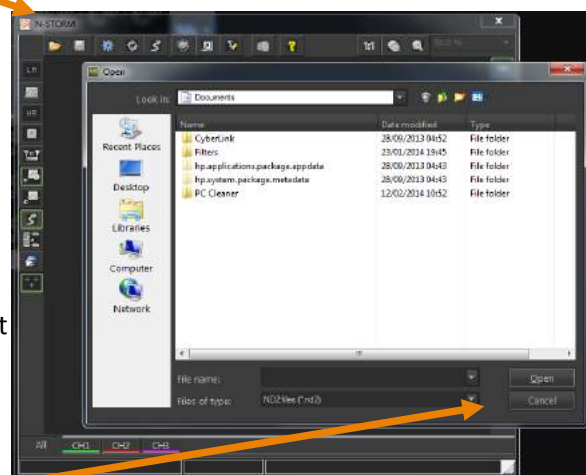
Select Analysis Gui from the bottom of the N-STORM Pad



In the GUI using the folder icon find the raw data

Select the required file and load the associated Molecule list

(If data are moved the raw data ND2 file and the .bin and .txt document which contain info about molecule position and drift must be moved together. Otherwise the localisations will be lost.)

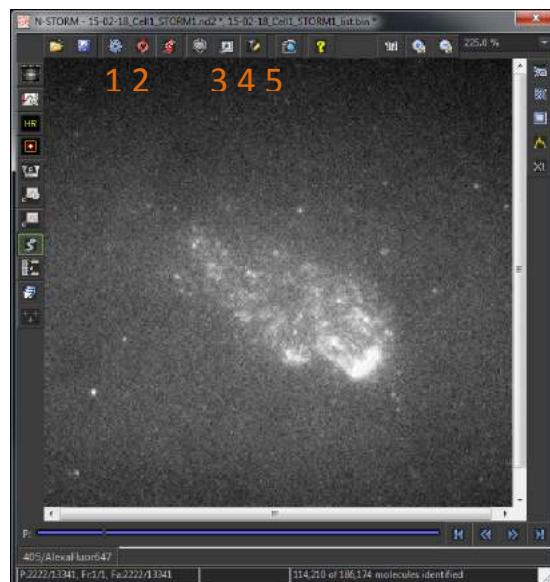


Press Open

In the STORM analysis GUI, The raw image is opened

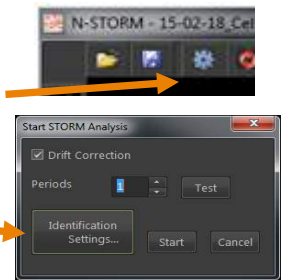
N-STORM GUI: Quick guide to useful icons

- (1) Identification Parameters (blue cog) defines what a particle is or isn't
- (2) Red arrows = Start STORM analysis
- (3) Display settings are white rectangle with arrow
- (4) Filter settings is the sieve
- (5) Capture image snapshot for easy export
- (6) Resolution / localisation accuracy, right click and select Properties



The 2D fitting is centroid fitting like QuickPALM (2)

When analysing a Storm experiment the ID settings need to be carefully set up. Press the blue cog to open the Identification settings GUI. In the 'Start STORM Analysis' dialogue box press **Identification Settings** to optimise how the photoswitching molecules are identified.



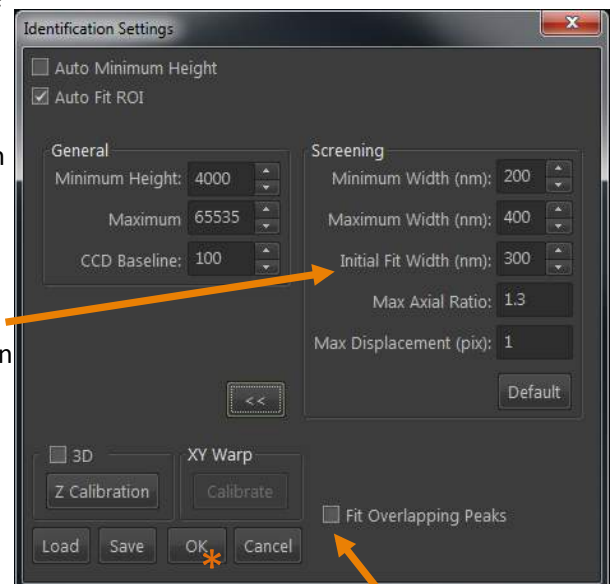
It is recommended that the ROI is autofitted. To define the parameters for autofitting press >> (*)

Expands showing the Screening menu.

This allows definition of the minimum and maximum width expected of a fluorophore blink

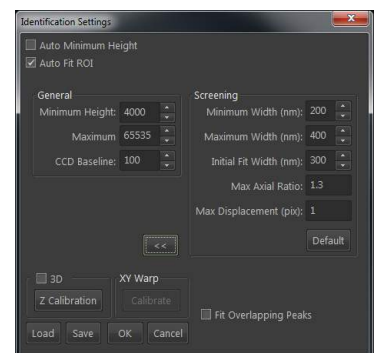
The initial fit width (i.e. how large the expected diffraction limited blink is) and the expected discrepancy in the ratio in the x and y plane (fit tolerance).

The centroid fitting cannot cope with overlapping blinks from 2 or more fluorophores and rejects them.



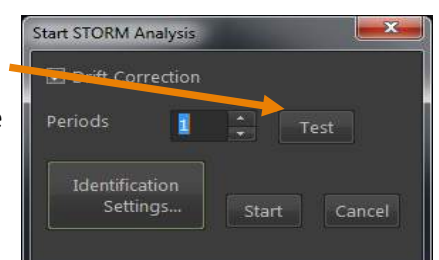
If the structure is filamentous or densely labelled the centroid fitting will not work. Instead overlapping pixels can be fitted by checking the box. *This is multiple emitter fit code written by the Zhuang lab (3)*. This is slower.

For 3D STORM analysis the Auto Fit ROI should be checked. The Maximum width should also be adjusted, initially try 800 and an axial ratio of 3



Once the ID settings have been set up they can be tested to see how well they perform by pressing **Test** in the **Start STORM analysis** GUI

It is recommended that a period (i.e. frame) is chosen in the middle of the experiment as there will be quite a few images which are too saturated with blinks at the beginning to localise.



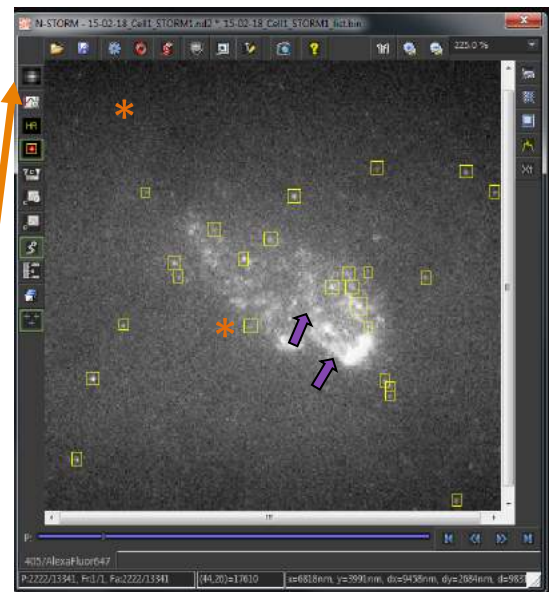
An example of localisations is shown (right)

False positives are indicated. (*)

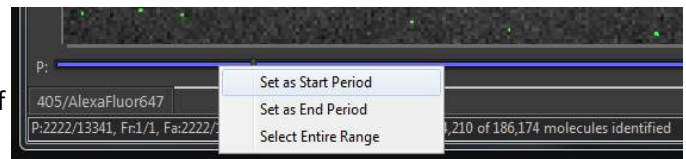
These can be removed by adjustment of the Identification setting parameters

False negatives (i.e. blinks which aren't identified by the system ➡) can be corrected for by refining the ID settings

N.B. To remove the yellow squares the icon on the LHS menu with a yellow square around it can be pressed.



The data to be analysed can be cropped in the analysis window by right clicking at the bottom of the image on the frame and selecting the **start** and **end period** to analyse



To start the STORM analysis once the experiment is complete press **Start** in the **Storm Analysis** gui



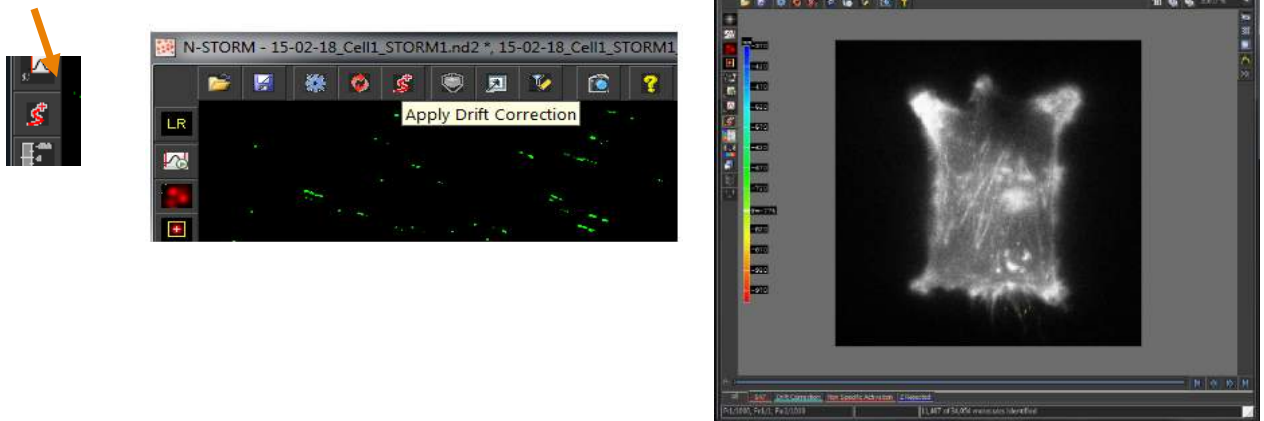
This will automatically start processing the image and generate an output image which is an overlay of the low resolution STORM image and the high resolution fitting.

Post processing the 2D/3D STORM image

The processed STORM image (right) consists of dots, these indicate blinks and their size and shape depend on the localisation accuracy which can be adjusted.

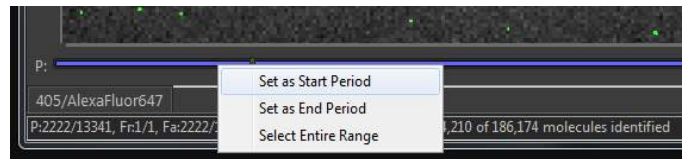
(1A) Correct system drift: Drift can be corrected by pressing the drift correction button. If the system has fiducials + these have been detected these are used. Otherwise an autocorrelation algorithm corrects drift

The drift correction can be applied or removed once calculated using the snake icon in the LHS menu



(1B) If the first few frames are saturated these can be removed from the analysis so only the photo-switching events are analysed

The data to be analysed can be cropped in the analysis window by right clicking at the bottom of the image on the frame and selecting the **start and end period** to analyse



(2) Remove false positive localisations:

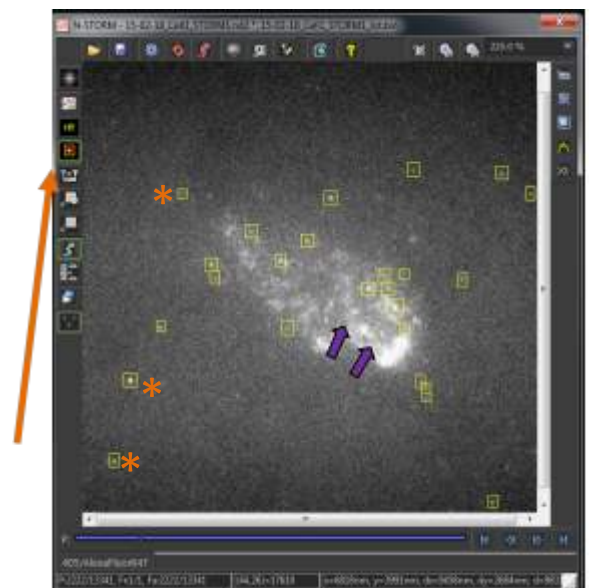
An example of localisations is shown (right)

False positives are indicated (*)

These can be removed by adjustment of the Identification setting parameters

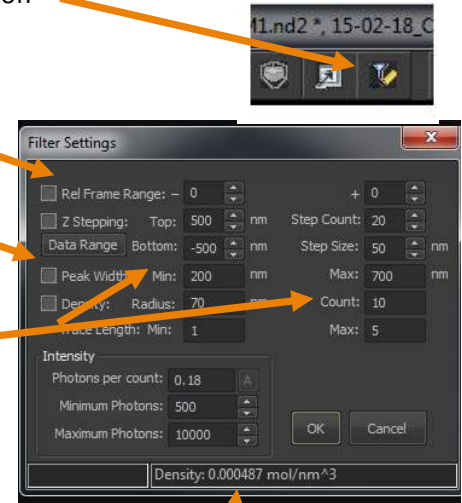
False negatives (i.e. blinks which aren't identified by the system (Purple arrow) can be corrected for by refining the ID settings

N.B. To remove the yellow squares the icon on the LHS menu with a yellow square around it can be pressed.



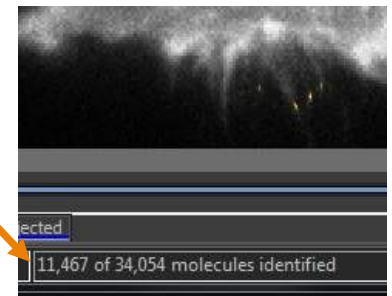
The molecules selected can be refined using the filter settings button

- Here the relative frame range can be set.
- The number of localisations can be refined by adjusting the Density parameters.
- The radius is the search radius for a localisation to be considered 'real', setting this to 5x the resolution can help.
- Also altering the count which is the number of blinks found within the search radius can be adjusted. This means that only areas where there is a large amount of blinking, (e.g. an epitope which has bound to an antibody with a dye on it / a.k.a. Real staining) will be localised.
- For 3D the Step count and range can also be controlled



The number of molecules shown in the final image vs blinks identified is shown in the menu at the bottom of the window.

Antibodies and dye molecules can stick to glass and dirt / crud on the coverslip can also cause spurious switching. Adjusting these parameters reduces the number of identified molecules (blinks) shown. This is acceptable as some are false positives. The number of localisations shown is indicated at the bottom of the results GUI



(3a) Multicolour STORM - analysis Correct the colour registration in the image

To reconstruct the data everything is the same as for single colour imaging except that the spectral registration of the colours needs to be carried out.

In the STORM analysis GUI the spectral registration can be carried out by using the spectral alignment GUI

When the three crosses are separate there image is not registered. At the last calibration chromatic aberration of 100nm on this system was noted.

Click the icon and all of the crosses move together, the image is now spectrally re-registered.



Post processing 3D Multicolour images will be discussed below:

(4) Determine localisation precision (standard error of fitting) to do this the photons per count should be entered into this box. They can be calculated the following information:

Photons per count calibration (filter settings STORM GUI)

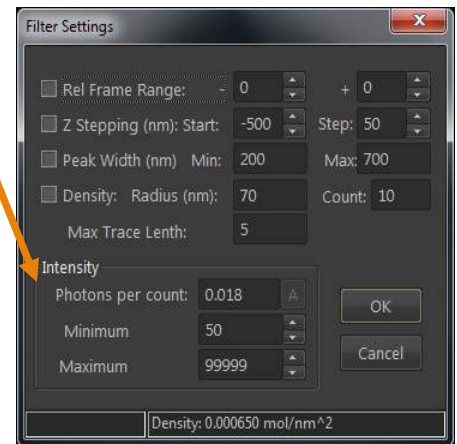
Use Andor DU897 camera settings:

- EM gain 17MHz at 16-bit
- Conversion gain of 3
- Photons per count = 4.85/EM gain

So EM gain of 100 = 0.0485

So EM gain of 200 = 0.024

So EM gain of 300 = 0.016



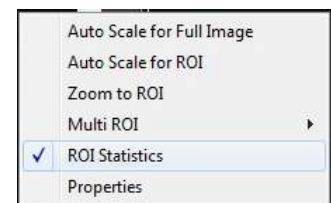
(5) Export all the information about localisations right click in the STORM imaging analysis menu

It is essential when images are reported in the literature that they contain not only a scale bar but also information about the error of localisation as this depends on how much light is collected. This data also should be kept in case of editors questions

NB Error of precision must not be used until the calibration for photons per intensity count has been entered into the filter settings menu as an incorrect value will invalidate the equation (see Betzig Science 2006 for more info about how the maths works)

The information about the STORM experiment can be saved by right

clicking in the STORM image and choosing properties.



Information about the Experiment, the positions of the molecules and statistics is kept here. This file should be saved for each experiment.

Visi...	Channel Name	X	Y	Xc	Yc	Height
Visible	405/AlexaFluor647	8868.2	12603.8	9075.5	12635.1	7613.22559
Visible	405/AlexaFluor647	14875.1	15911.4	15082.4	15942.7	6887.76855
Visible	405/AlexaFluor647	19808.0	16687.2	20015.3	16718.5	9152.69629

(6) Determine Localisation Precision for a given area

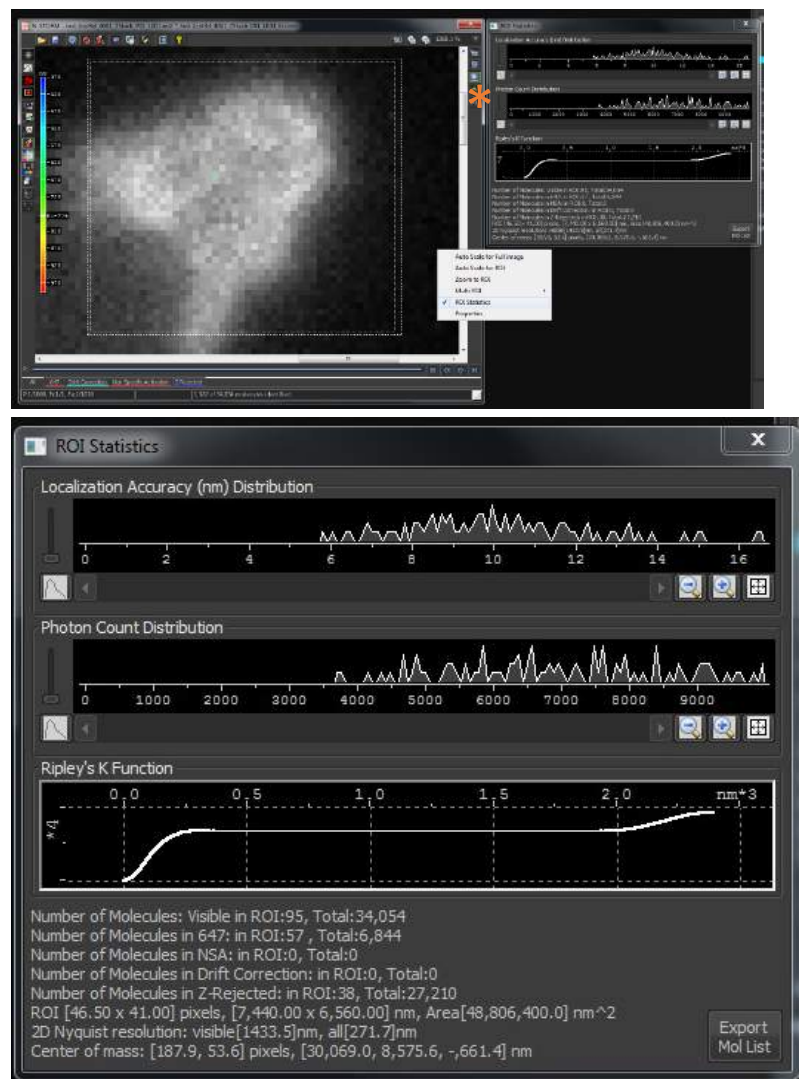
To find out the localisation precision for a given area click on the ROI button (*) and draw an ROI.

Right click in the STORM image and choose **ROI statistics**. The Statistics tab pops out.

Its possible to have Multiple ROIs as well to compare statistics, choose this option.

The menu with ROI statistics will appear. Ensure that the Molecule list is exported if data are for publication.

This shows the localisation accuracy, photon count and clustering coefficient (Ripley's K function). See Nikon Technical note about this.



(7) Exporting publication quality STORM images

RHS icons which may be useful for localisation precision and image display



- Top shows or hides the scale bar
- Next down puts a grid on the image for looking at intensities
- The next one creates an ROI
- The yellow graph shows the intensity of a blink in raw and reconstructed images

Top menu icons which may be useful.



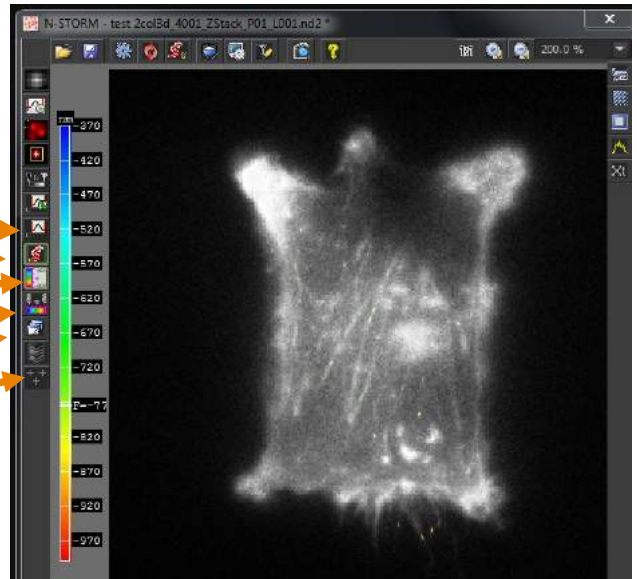
- Disk Icon saves the data
- The camera icon does a screenshot of the image

Post processing the 3D multicolour STORM image

The output image shows the Z information which was included and excluded. The Axial (3D) drift needs to be corrected using drift correction:

LHS Icons:

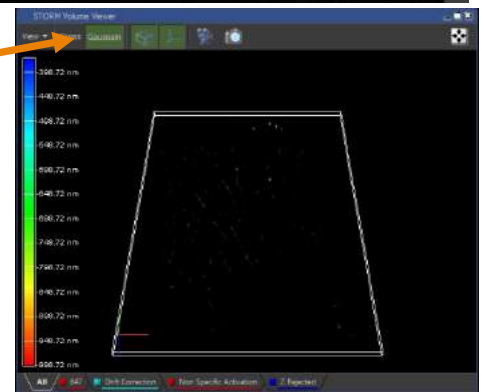
- 3D drift correction
- For depth coding the 3D image,
- For controlling 3D in time
- Batch process
- 3D Volume viewer
- Colour correction of the 3D image



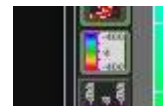
3D display of the STORM image can be visualised by pressing the 3D volume icon

(top menu)

- In the 3D viewer you can control the display of the rendered image. The camera icon allows the output to be stored.
- The camcorder allows a movie to be taken.
- The crosses for localisation can be viewed.
- Different planes (X, Y or Z can be visualised using the View tab).



To change the depth coding in the image Right click the Depth coding icon

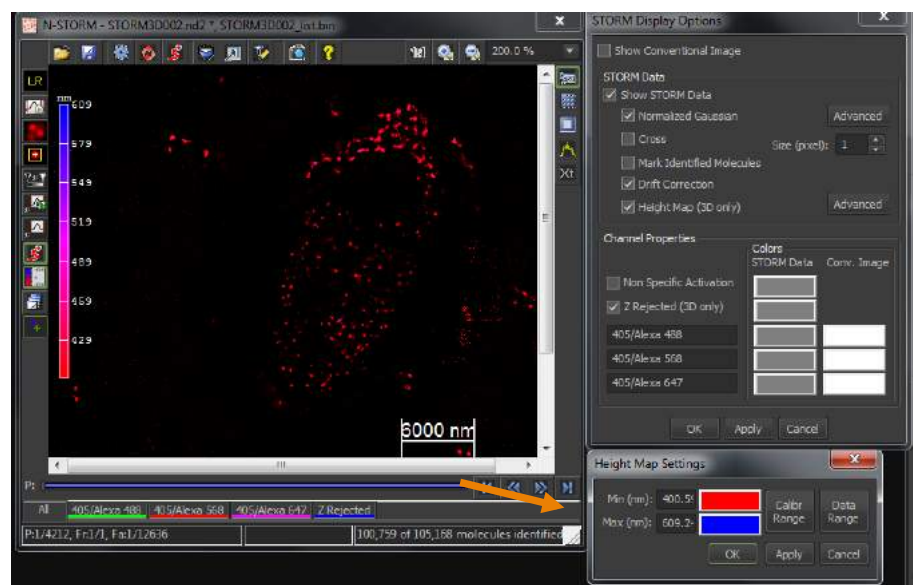


Here Height map can be selected/
By pressing the Advanced tab the look up table for the height map

By changing the Min + Max and colours in the box the 3D rendering colours alter and a scale coding depth intensity (LHS) appears

The z rejected blinks (because they were a funny shape) are shown or hidden here and the height map can be toggled on and off.

Data should be exported in the same way as a 2D imaging



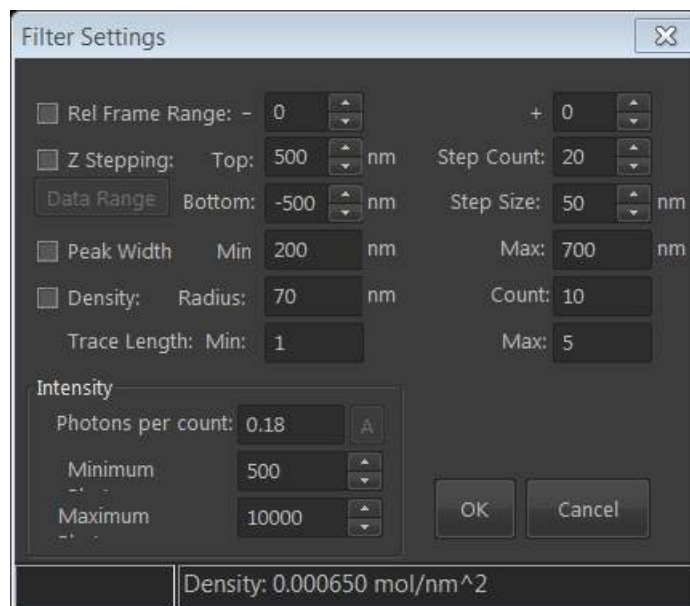
N-STORM Analysis Tools:

Filter Settings (Density)



Density: This is a density based filter whose main purpose is to clean up the background localizations outside of the structure of interest. It is implemented using publicized DBSCAN algorithm. The algorithm groups points into clusters by exploring their topological relationships. The filter can be enabled or disabled with its check box. Just like its underlying algorithm it requires two parameters: Radius and Count.

Radius is the max neighborhood search radius. Count is the min count for a group of points to form a cluster. Points that don't form clusters of required density are considered noise and get hidden. Density filter is applied last. This allows all other filters to form the input for the density filter. This filter is interactive. Its effect can be visible without closing Filter Settings dialog. Radius and Count can be changed by either typing new values followed by Enter or via mouse scroll wheel while the cursor is hovering over the corresponding edit box. The adjustment range and acceleration options can be found in the tooltips.



The status bar at the bottom of the Filter Settings dialog pertains to Density Filter. The right pane displays the density calculated as the ratio of the current count over the round area for 2D data (or over the sphere volume for 3D) with current radius. Double clicking the right pane toggles the area or volume units between nanometers and micrometers (squared or cubed correspondingly). As the radius and count are adjusted the density is recalculated.

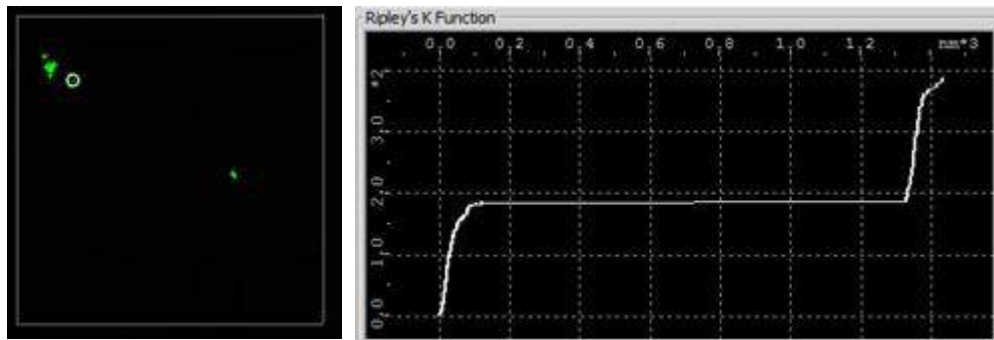


N-STORM Analysis Tools:

Ripley's K function

Displays a cumulative histogram of distances between molecules in ROI population.

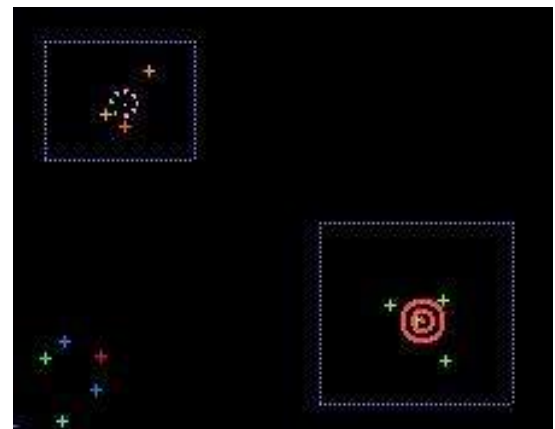
Ripley's K function is useful to explore cluster topology. When molecules form clusters, Ripley's K function forms horizontal plateaus and steep rises (staircase look). For example, in the simplest case of two clusters, Ripley's K function has one horizontal plateau.



The horizontal axis of Ripley's K function is the distance between molecules in a given pair. The vertical axis is the number of pairs in the population with distance not exceeding given. The first vertical rise on the left side comes from a number of close by neighbor molecules within each cluster. The left edge of the horizontal plateau tells that there are almost 200 distances of less than 100 nm. This implies the size of clusters. As the distance increases further, the number of such pairs does not increase until the distance reaches the distance between clusters. At that point the count begins to rise, because larger distances between molecules of different clusters begin to contribute. Therefore the right edge of horizontal plateau suggests the inter cluster distance. Mouse cursor position in graph units is displayed in the bottom right corner as the mouse hovers over the graph area.

Center of Mass distance measurement

The distance between two Centers of Mass can be measured with the help of Multi ROI feature. To do this, add at least two ROIs to Multi ROI. Then double click on the center of mass circle of one of the ROIs. This sets it as a measurement datum and marks it with two concentric red circles. As the mouse cursor hovers near another center of mass the status bar shows relative distance between the two. Horizontal and vertical components are also displayed.



Useful publications



Method for co-cluster analysis in multichannel single-molecule localisation data

J  r  mie Rossy, Edward Cohen, Katharina Gaus , Dylan M. Owen 

<http://link.springer.com/article/10.1007%2Fs00418-014-1208-z>

Three-dimensional superresolution colocalization of intracellular protein superstructures and the cell surface in live *Caulobacter crescentus*

Matthew D. Lew^{a,b,1}, Steven F. Lee^{b,1,2}, Jerod L. Ptacin^c, Marissa K. Lee^b, Robert J. Twieg^d, Lucy Shapiro^c, and W. E. Moerner^{b,3}

Author Affiliations 

Contributed by W. E. Moerner, September 5, 2011 (sent for review July 25, 2011)

<http://www.pnas.org/content/108/46/E1102.full>

Additional information and scripts for the freely-available ThunderSTORM package:

<https://github.com/cleterrier/ChriSTORM/blob/master/README.md>