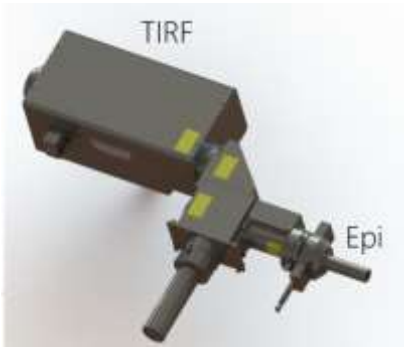
Nikon Training for the Ti2 NSTORM

Contents

STORM System components

Ti2 – controller and microscope main body
Laser Bed
Cameras
PC

Ti2E Microscope main body



Ti2 Controller

LUN-V Laser Bed

Power button is on the back and key on the front. Individual lasers can be turned on and off using the individual buttons on the front

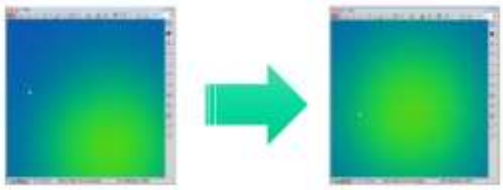
Camera

Power button is on the back

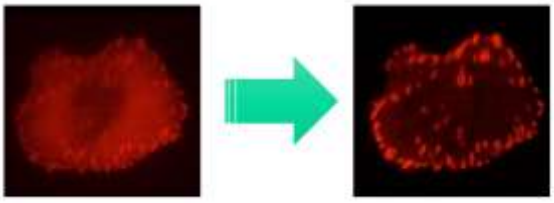
Z8 PC

Setting up the Microscope for TIRF

1. Clean lens and sample
2. Place sample on stage and focus. Check correction collar settings too
3. Remove sample and clean lens again
4. Focus and centre laser in back focal plane



5. Centre illumination in sample plane using a fluorescent chroma slide. This may require centering the fibre entering the illuminator although this should only need to be done at install.
6. Put sample back on and adjust TIRF angle until TIRF is achieved

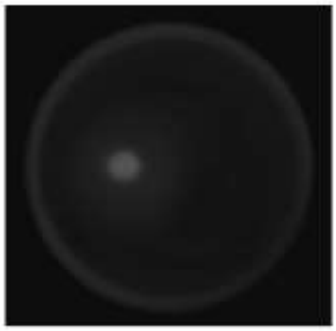


Focusing the laser at the back focal plane

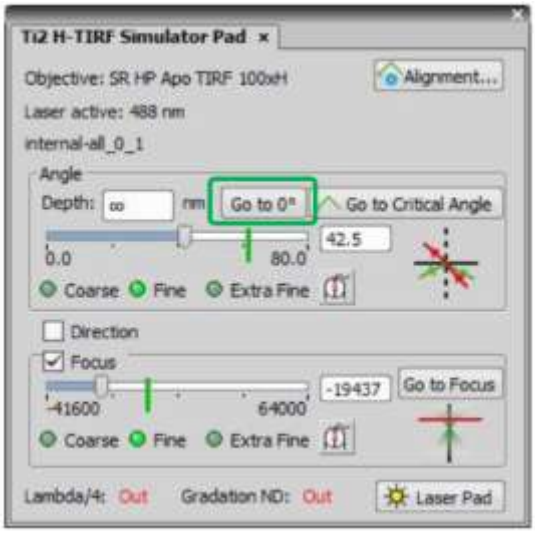
The Ti2 has the option of putting a camera on the binocular side port and inserting the bertrand lens to image the back focal plane. This means the laser can be centred and focused safely using a camera image.

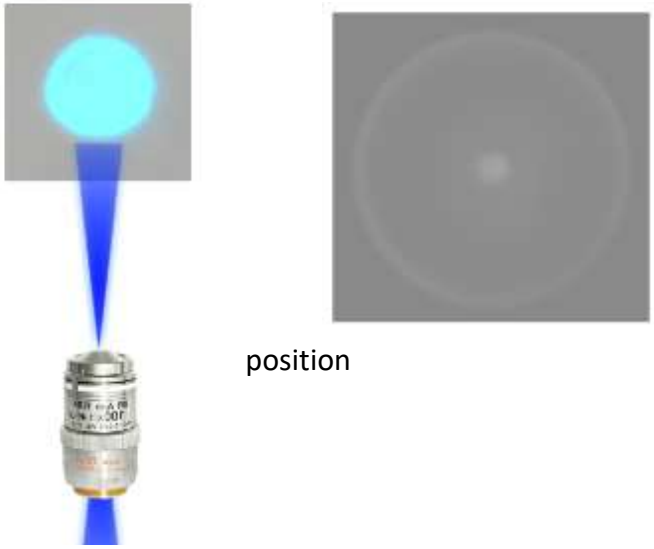
1. Without a sample on the stage, turn on the laser being careful to watch where the laser light is pointing. The interlock lid should be over the sample to ensure you are protected from a stray beam of light.

Image at the back focal plane

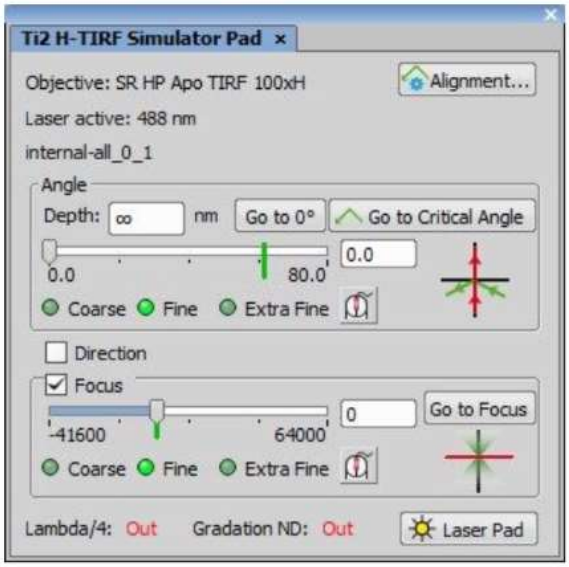


2. The HTIRF pad allows you to simply set the angle of the beam to centre the position





3. Once centered, you can focus the beam using the H TIRF pad. If the bean is centered at the back focal plane the beam is collimated and would give a small focused spot on the ceiling. Again you can simply view the focused spot on the back focal plane using the camera and the Bertrand lens.

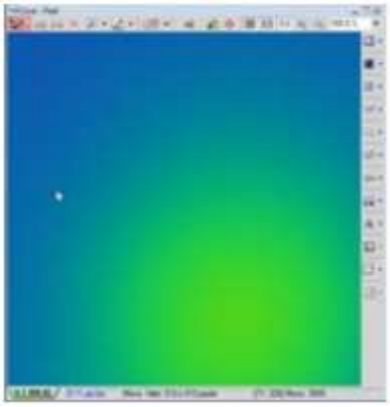


4. Once the beam is focused you can check the centering of the laser light at the sample plane.

Focusing the laser in the sample plane

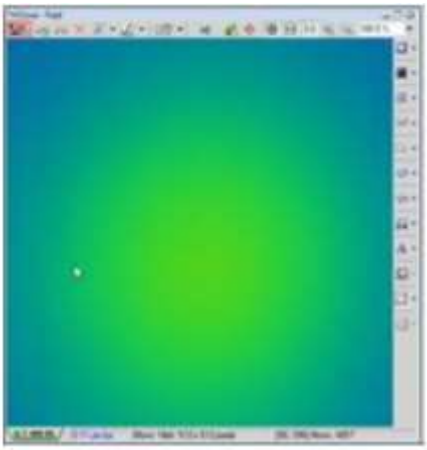
Using a chroma slide check the evenness of the illumination at the sample plane. Set the Look up table (LUT) to rainbow dark to clearly see the centre of the bright spot.

off-centre

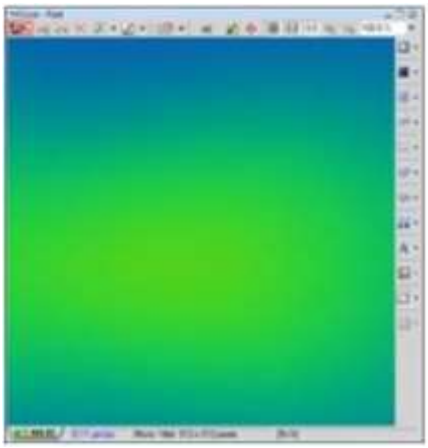


Use allen keys in opposite pairs to move the laser left/right/up and down. This should only need to be done once at installation although the use of alternative STORM lenses may mean this is needed to be checked more frequently.

centred

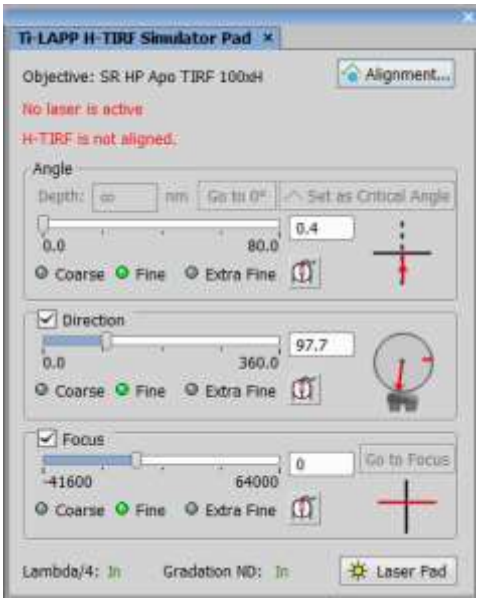


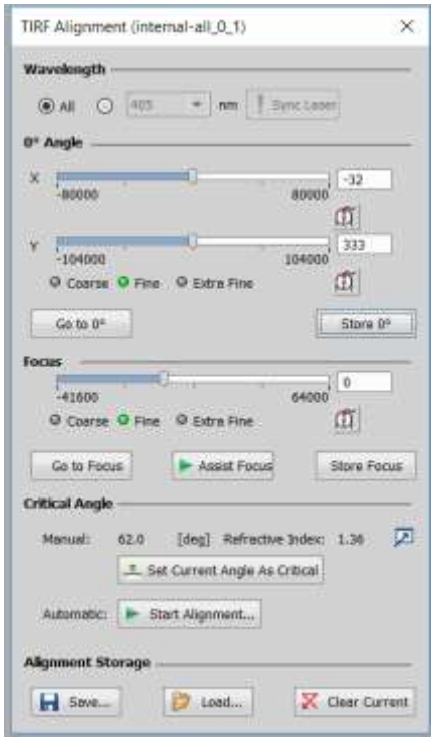
When in TIRF



Setting the TIRF angle

The TIRF angle is adjusted using the HTIRF pad and once set can be saved. The beam can be directed anywhere in 360 degrees and the TIRF angle set for multiple directions. There is also an auto alignment option with the H TIRF.





Once in TIRF you should see both the excitation and emission beam at the back focal plane

Epi Fluorescence

TIRF

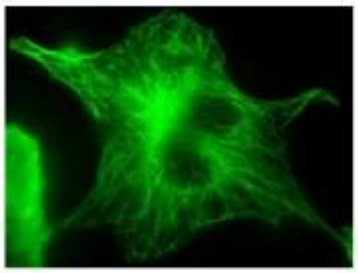


Image at the sample

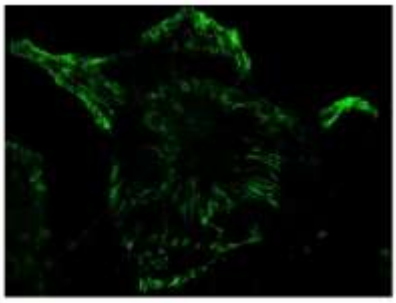
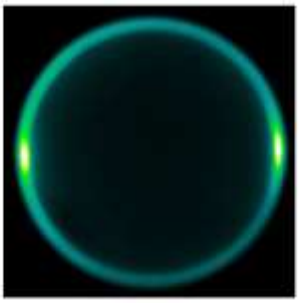


Image at the back focal plane



STORM Software

Calibration

There are three image acquisition modes, and calibration with fluorescent beads is required before image acquisition.
1) 2D-STORM: Image acquisition with position information in the XY directions.
2) 3D-STORM: Image acquisition with position information in the XYZ directions. Use a cylindrical lens.
3) Z-Stack: Image acquisition with Z stack.

Correction by [Z Calibration] and [XY Warp]

When performing 3D-STORM or Z-Stack, it is necessary to perform calibration in advance so that the position in the Z-axis direction is correctly analyzed.

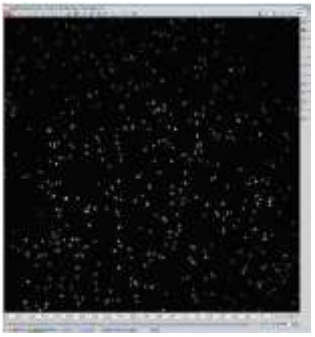
[Z Calibration] correlates the “position in the Z-axis direction” and “the ellipticity of the bright spot image out of focus”.

When with multiple channel, it is necessary to correct the color shift caused by chromatic aberration in the XY direction (2D) and Z direction (3D).

[XY Warp] corrects color misregistration in the XY direction, and [Z Calibration] corrects color misregistration in the Z direction in addition to the above analysis.

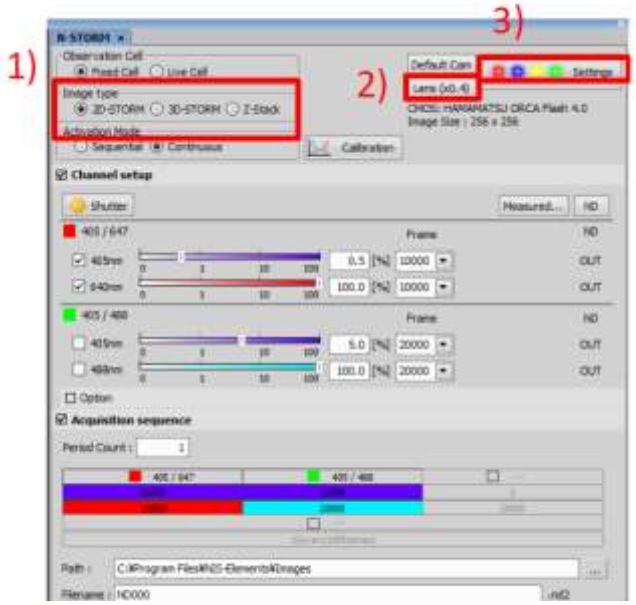
Sample to be used
TetraSpeck Microspheres, 0.1um Fluorescent

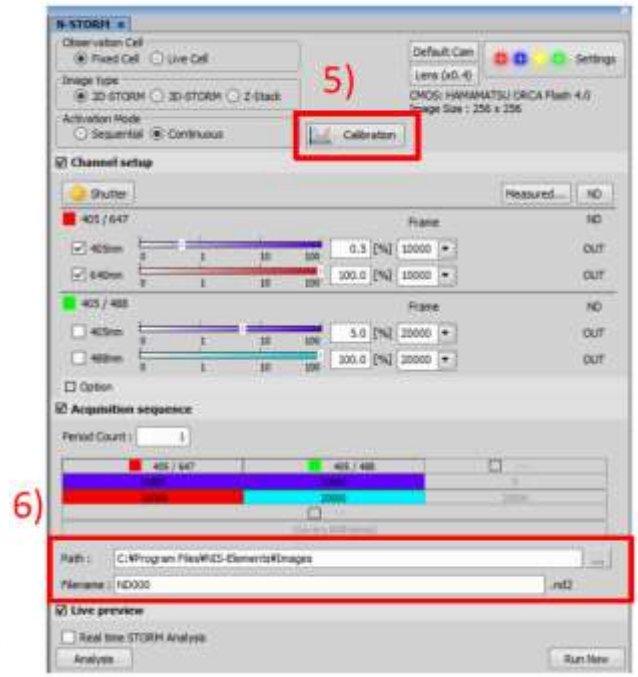
2D STORM Calibration



1. Focus the bead specimen.
Select the place where the over 50 beads are not lumpy and are scattered.

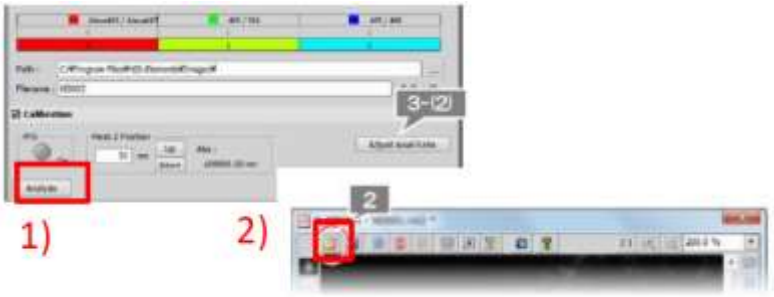
2. Start calibration.
1) N-STORM control screen
Image type: 2D-STORM
2) Set [Lens]
Lens (x1): Manual STORM port
Lens (x0.4): Moto STORM port
3) Set [Setting]
Set the laser and channel color in the Sequence tab as follows, and press [OK].
Ch1 = Activation: 405 Reporter: 647
Ch2 = Activation: 405 Reporter ： 568
Ch3 = Activation: 405 Reporter: 488
4) Focus on the beads image
5) Press the [Calibration] button.
6) Set the save destination and file name.
Path: Use SSD for the saving folder.
Filename: Any
7) Confirm [Calibration] is checked.
8) Turn off PSF. The calibration cannot be performed unless PSF is turned off.
9) Click [Calibration] to start acquiring calibration data.





3. Load calibration image

1)Click [Analysis] to display the N-STORM analysis window.
2) Open the calibration file (ND2 file) from [File Open].

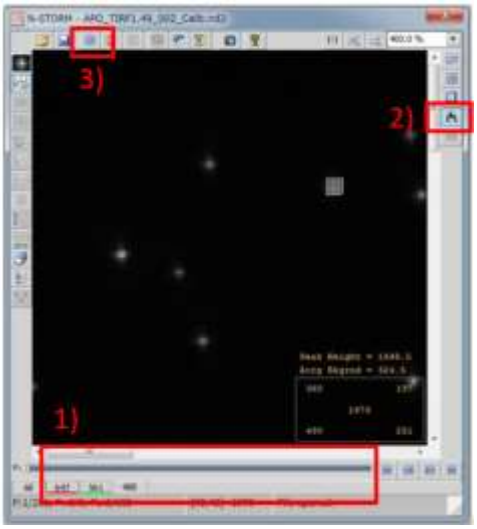


4. Setting conditions for bright spot analysis

1)Select the tab for each wavelength, and display the last image of the acquired image data.

2) Click [Use Peak Statistics] to display a 5 x 5 square box. Align the center of the box with the darkest of the bright spots and read the [Peak Height] value.

3) Click [identification Setting] to display the dialog box.

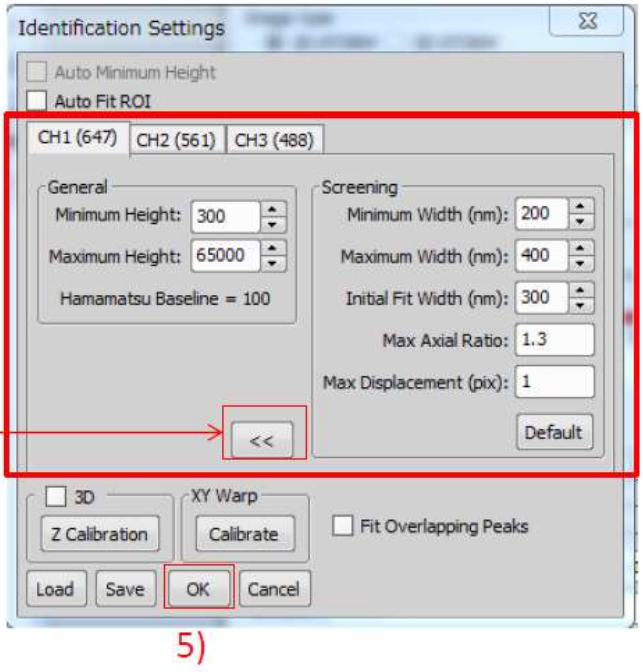


4) Set as follows for each channel in the dialog box.
Minimum Height: value from step 2)

Maximum Height:
sCMOS 65000, EMCCD 16000
Auto Fit ROI: Off

Click [>>]

Click [Default] to set all parameter to be initial value.
Minimum Width: 200
Maximum Width: 400
Initial Fit Width: 300
Max Axial Ratio: 1.3
Max Displacement: 1
5) Click [OK].



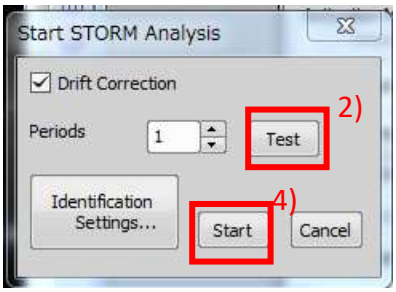
5. Molecule analysis

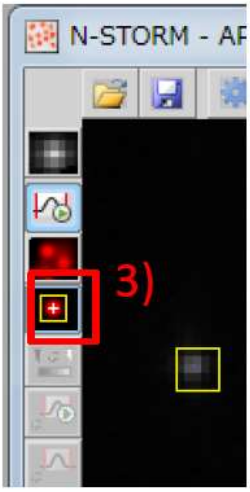
1) Click [Start STORM Analysis]

2) Click [Test] to Run test analysis

3) Turn [Mark molecules identified in current frame] on / off and confirm that the bright spots are correctly identified

4) Click [Start] to Start analysis



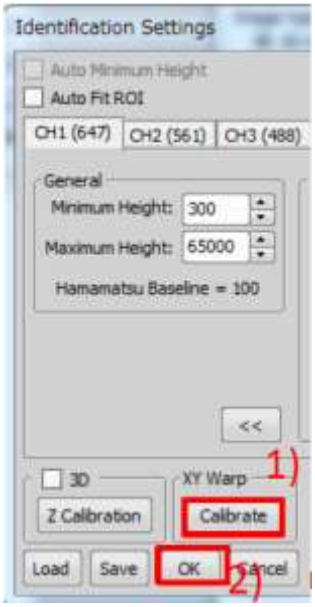


6. [XY Warp] Calibration

1) Click [Calibrate] of XY Warp from [Identification Settings]

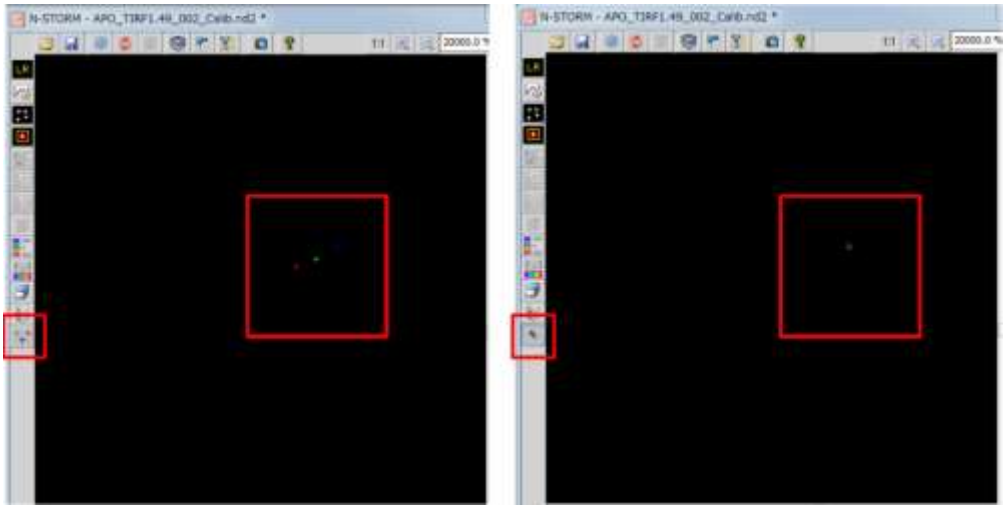
2) Click [OK] to close the dialog box

* The calibration value in the XY direction is saved as an internal setting of the N-STORM software and is automatically applied during subsequent 2D-STORM analysis.

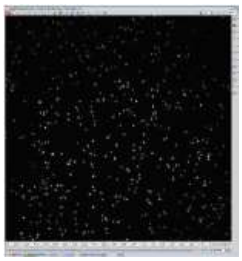


7. Result confirmation

Turn [Warped coordinates display] button on and off, and warp processing in the XY direction (correction of displacement in the XY direction due to chromatic aberration) is executed.



Calibration for 3D STORM



Calibrate both [Z Calibration] and [XY Warp].

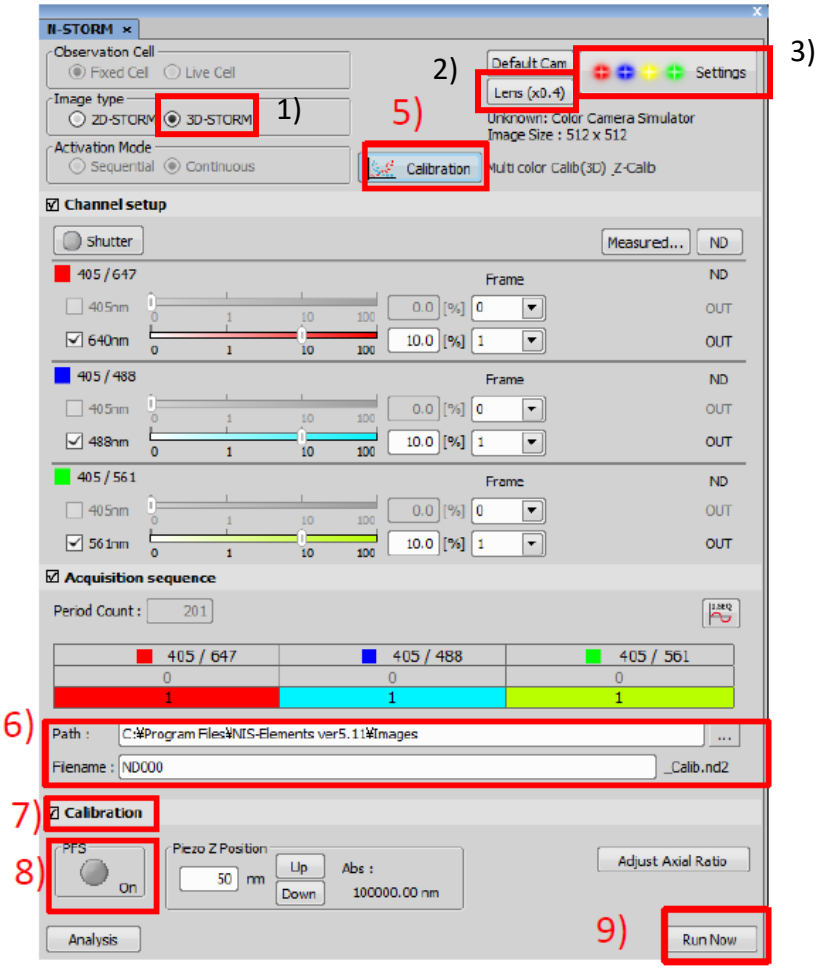
1. Focus the bead specimen.
Select the place where the over 50 beads are not lumpy and are scattered.

2. Start calibration.

1) N-STORM control screen
Image type: 3D-STORM

2) Set [Lens]
Lens (x1): Manual STORM port
Lens (x0.4): Moto STORM port

3) Set [Setting]
Set the laser and channel color in the Seque
Ch1 = Activation: 405 Reporter: 647
Ch2 = Activation: 405 Reporter ： 568
Ch3 = Activation: 405 Reporter: 488



4) Focus on the beads image

5) Press the [Calibration] button.

6) Set the save destination and file name.
Path: Use SSD for the saving folder.
Filename: Any

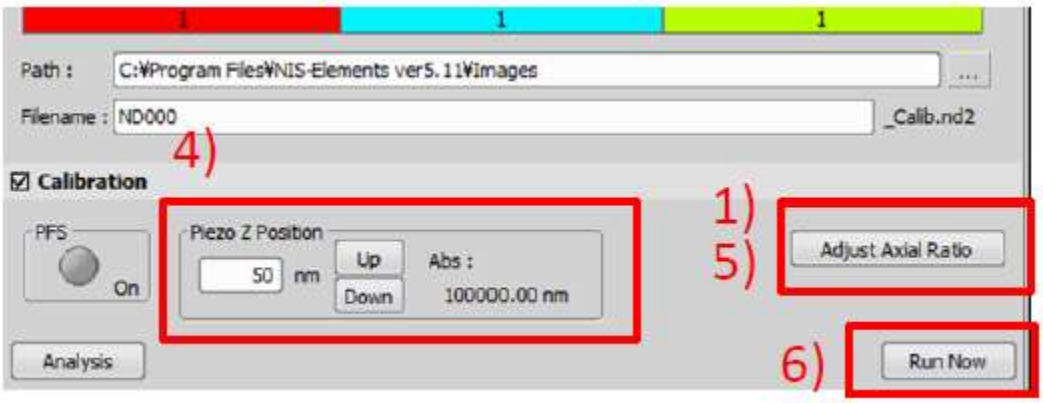
7) Confirm [Calibration] is checked.

8) Turn off PSF.
The calibration cannot be performed unless PSF is turned off.

9) Click [Calibration] to start acquiring calibration data.

3. Adjust the ellipticity of beads

1)Click [Adjust Axial Ratio] to display the N-STORM Detection Progress window and check the stability of the Ax Ratios ellipticity.



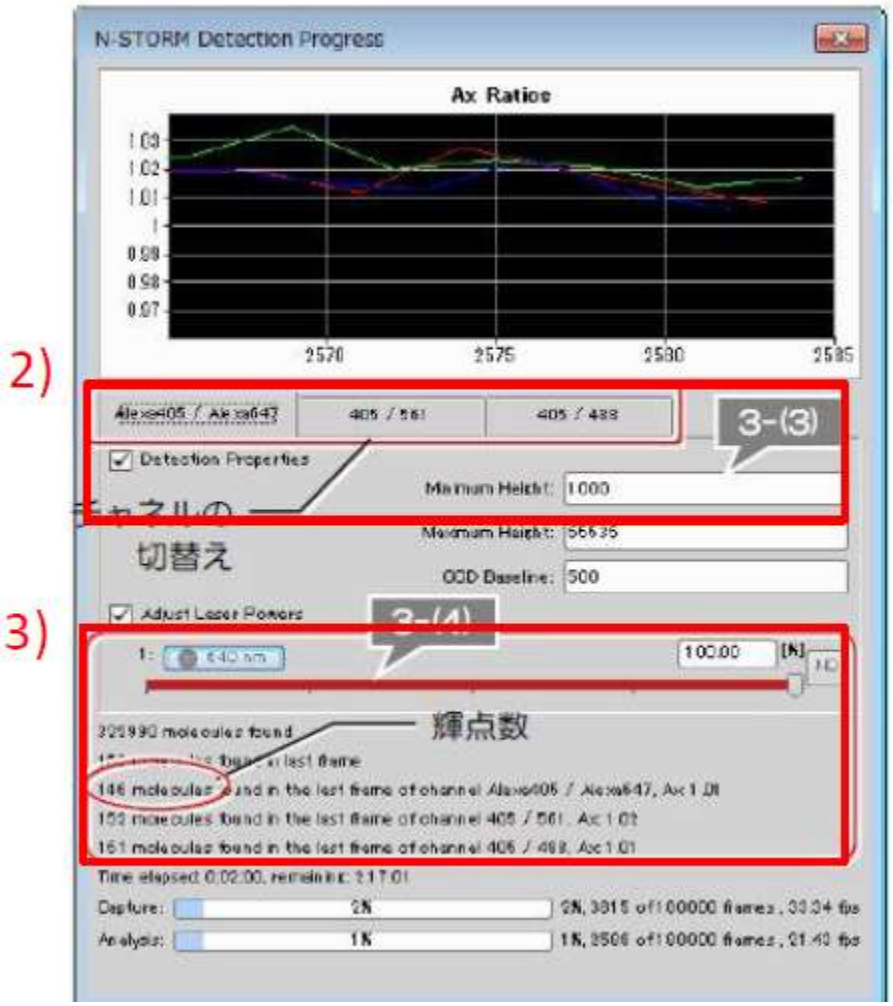
2) Adjust [Minimum Height] so that the number of detected beads in the field of view becomes almost the same at each channels.

3) Adjust the laser power so that the number of detected beads at each channels become the same.

4) Adjust the ellipticity of the beads. Click [Piezo Z] Up / Down in the N-STORM control window to stabilize the ellipticity ratio between 0.9 and 1.1 (approx. 1 minute).
* Do not change Ti / Ti2 objective Z controller

5) When the Axial Ratio stabilizes, click [Stop Axial Ratio] on the N-STORM control screen.

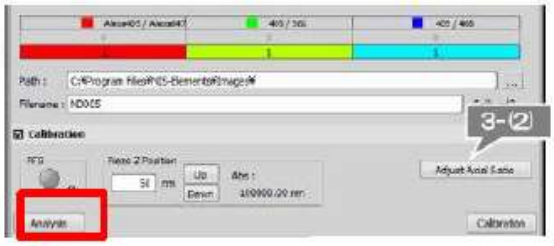
6) Click [Run Now] to obtain calibration data.



4). Load calibration image

1)Click [Analysis] to display the N-STORM analysis window.

2) Click [File Open] to open the image file (ND2 file).

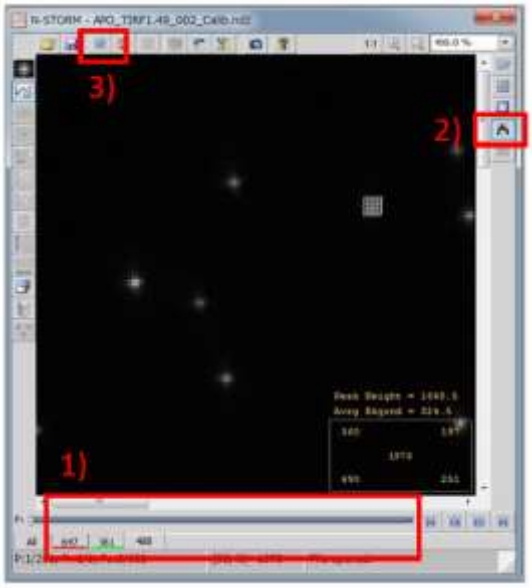


5. Setting conditions for molecule analysis

1) Select the tab for each wavelength, and display the last image of the acquired image data.

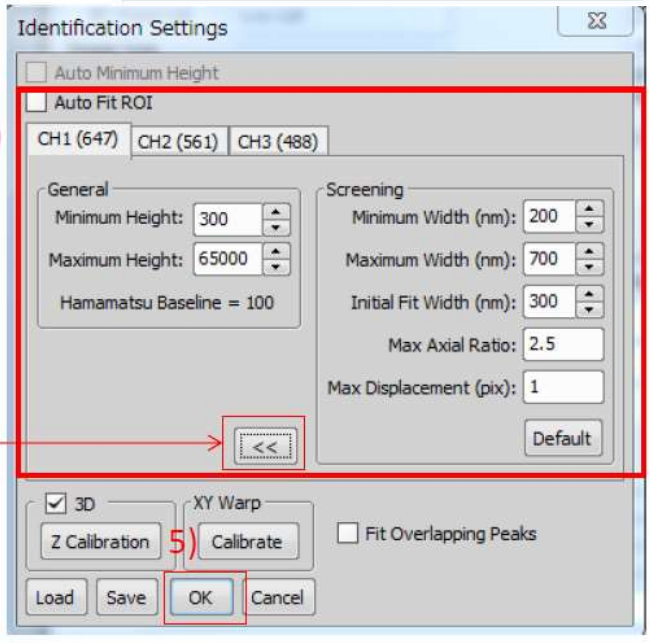
2) Click [Use Peak Statistics] to display a 5 x 5 square box. Align the center of the box with the darkest of the bright spots and read the [Peak Height] value.

3) Click [identification Setting] to display the dialog box.



4) Set as follows for each channel in the dialog box.
Minimum Height: value from 2) step
Maximum Height:
sCMOS 65000, EMCCD 16000
Auto Fit ROI: Off

Click [>>]
Set Screening to the default value with [Default].
Minimum Width: 200
Maximum Width: 700
Initial Fit Width: 300
Max Axial Ratio: 2.5
Max Displacement: 1
5) Click [OK].



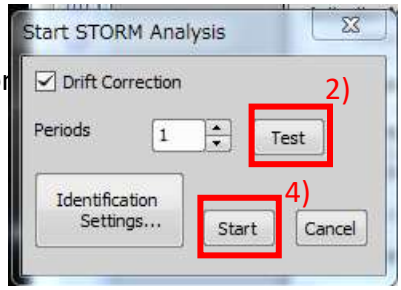
6. Molecule analysis

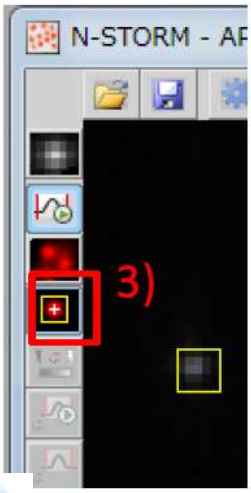
1) Click [Start STORM Analysis]

2) Click [Test] to Run test analysis

3) Turn [Mark molecules identified in current frame] on and check if bright spots are correctly identified

4) Click [Start] to Start analysis



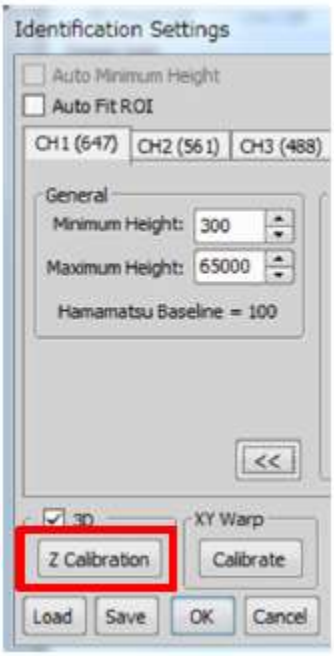


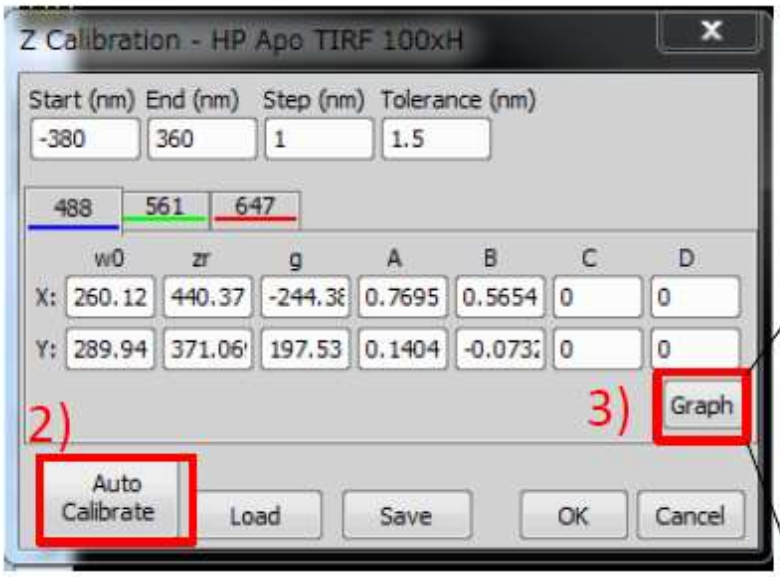
7. [Z Calibration] Calibration

1) Click [Z Calibration] from [Identification Settings]

2) Click [Auto Calibrate] and perform Z Calibration.

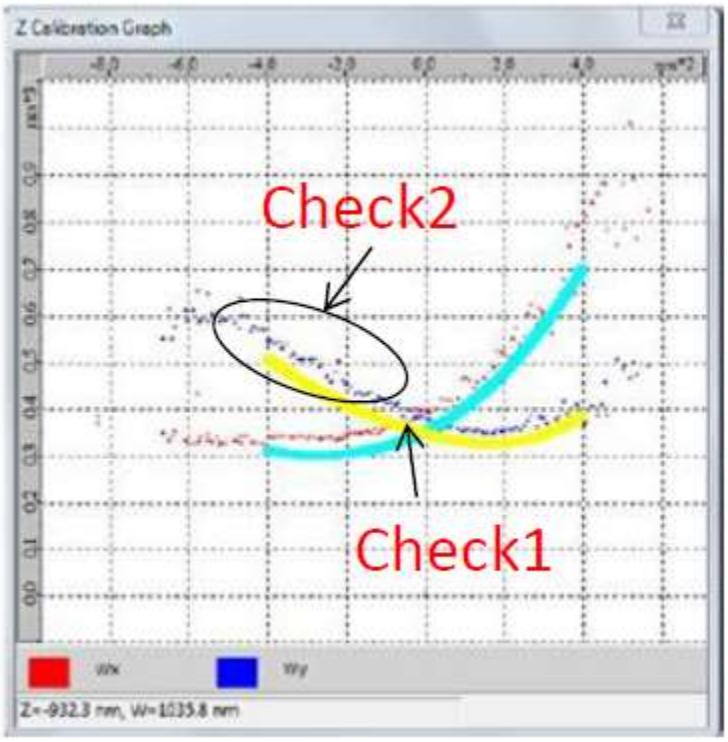
3) Check the results of Z Calibration using a graph.





Display the graph of [Wx / Wy vs Z] from [Graph] and check the following

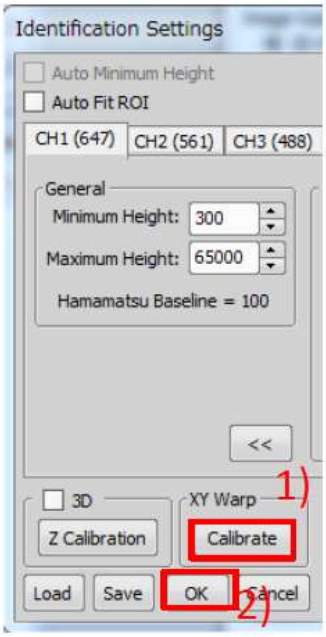
Check1>Wx and Wy intersect near 0 on the horizontal axis
Check2>There is no significant shift between Plot data (distributed points) and fitting



8. [XY Warp] Calibration

1) Click [Calibrate] in [XY Warp] from [Identification Settings]

2) Click [OK] to close the dialog box



STORM Acquisition Software

Open N-STORM “Acquisition control” window for condition settings.

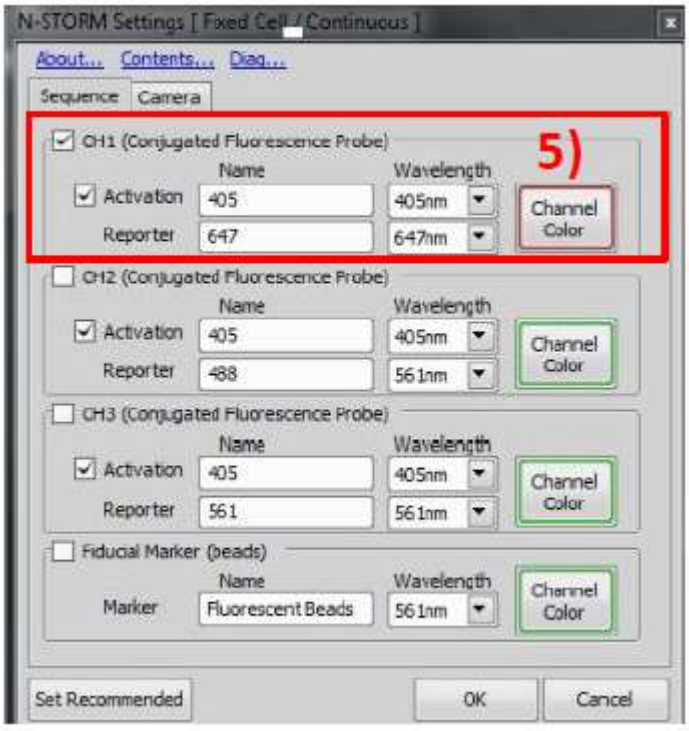
1） Select “fixed” or “live” cell.

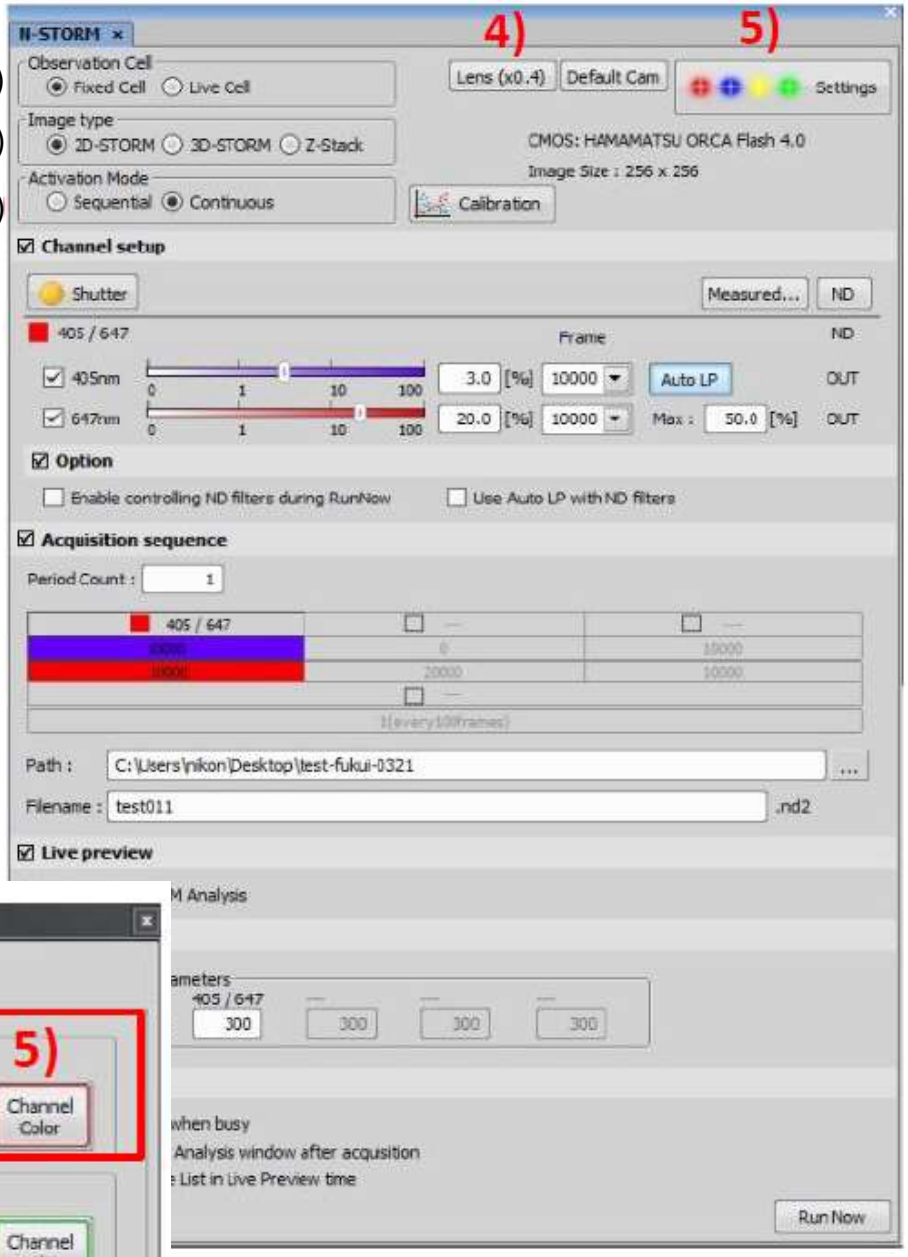
2） Select the image type.

3） Select the acquisition mode.

4) Set the lens（x0.4）.

5） Click the Settings button to open N-STORM settings window, set the channel name and wavelength.





6） Set the laser power values.

7） Set the number of frames.

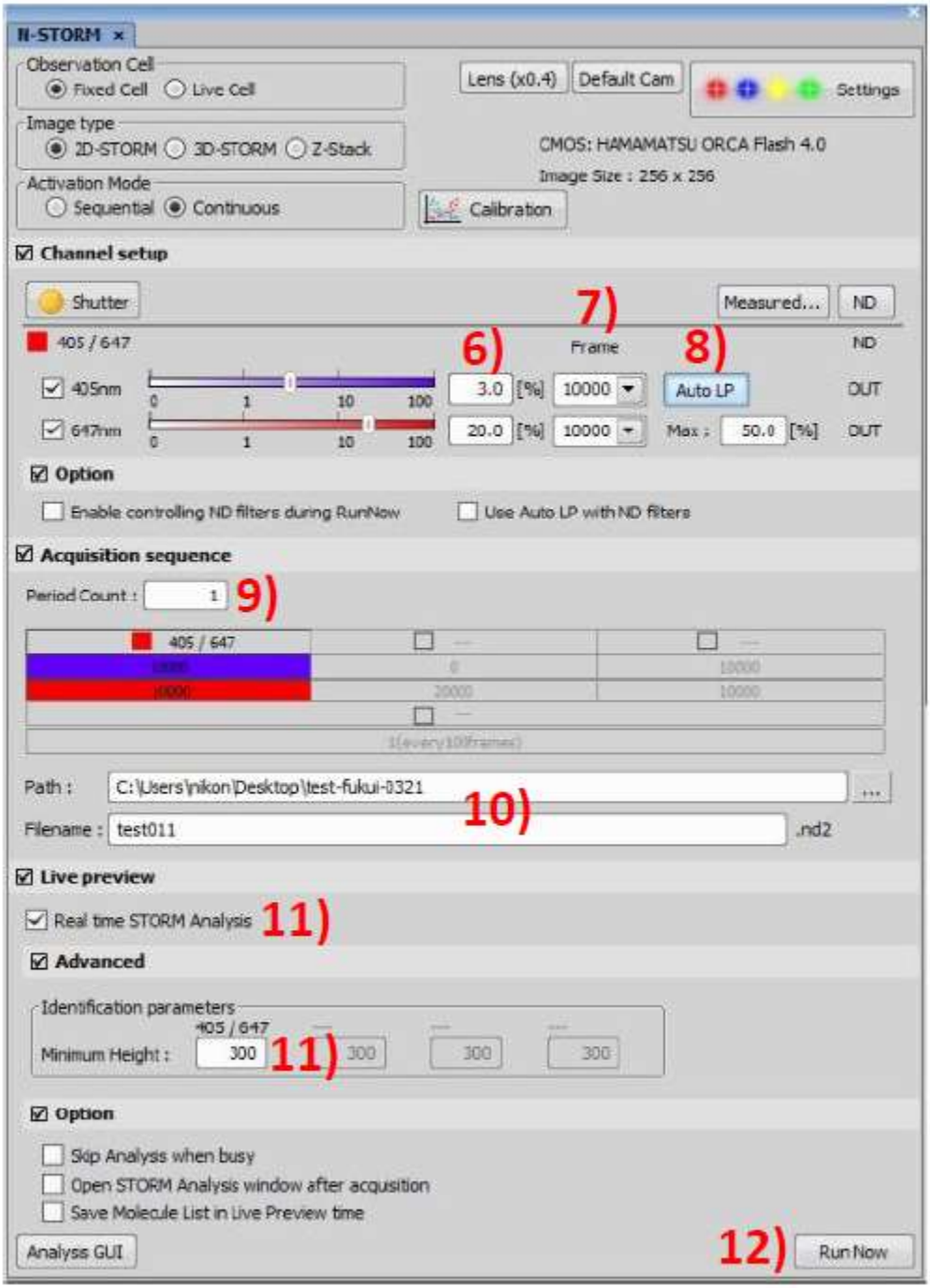
8） Turning on the “Auto LP” automatically adjusts laser power to maintain the number of detected molecules.

9) Set the period Count. “Frames” multiplies “Period Count” is the total frames for one channel.

10) Set the “Path” and “filename” to store ND2 file. Set the folder name of the solid state drive to store the STORM data for fast acquisition.

11) Turn on the “Live preview”.
Set the “Minimum height (over 300)” to preview the STORM image.

12) Click “Run now” to start the acquisition.



13) Other settings are the same as the conventional GUI.
Exposure time : Flash4.0 settings window
Laser beam angle for excitation: Ti2 N-STORM Pad
Image size : camera tab of ”N-STORM settings window”.

Molecule Option Window

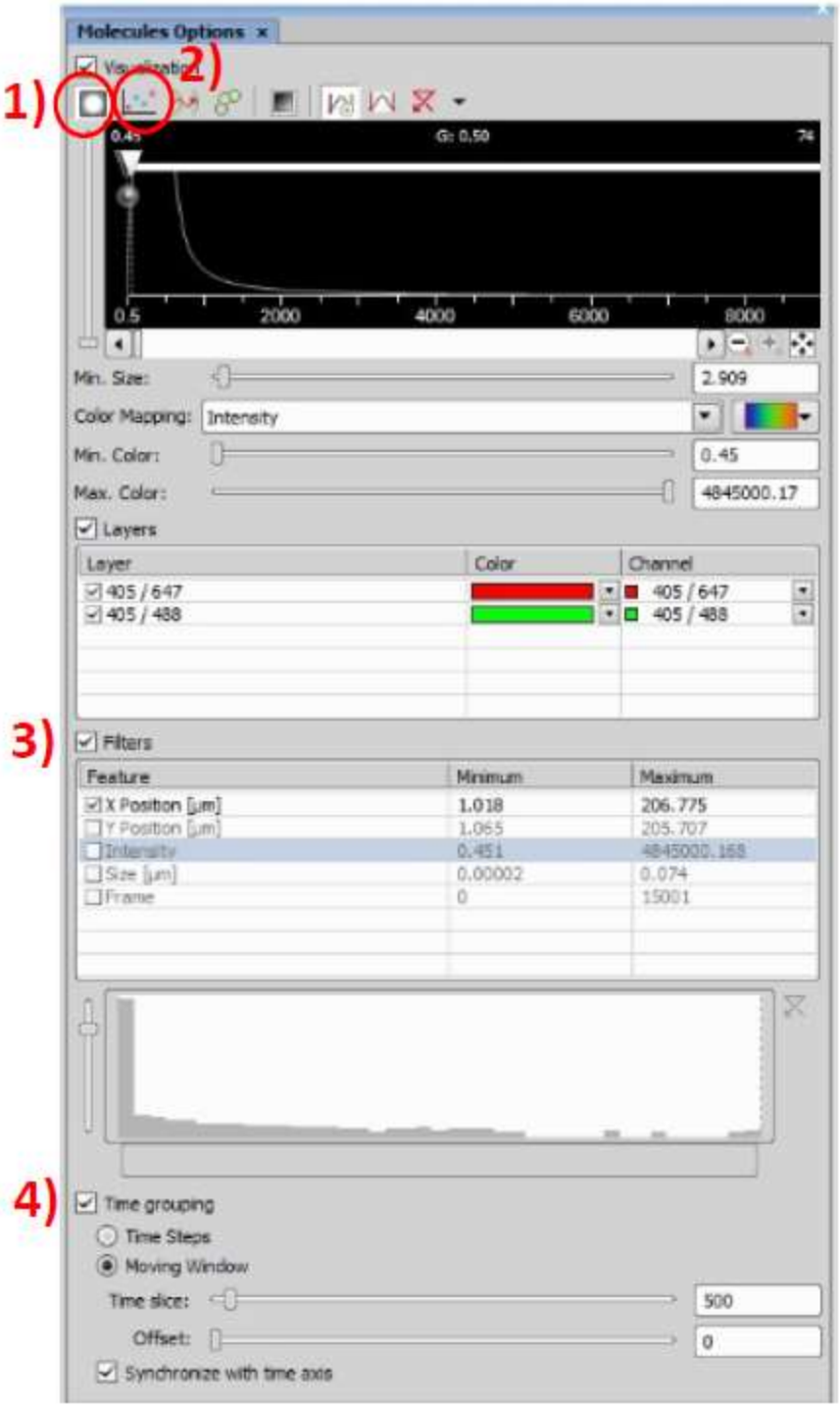
Open “Molecule Option window” of “Visualization control”.

1) Click the button to plot the Gaussian dots.

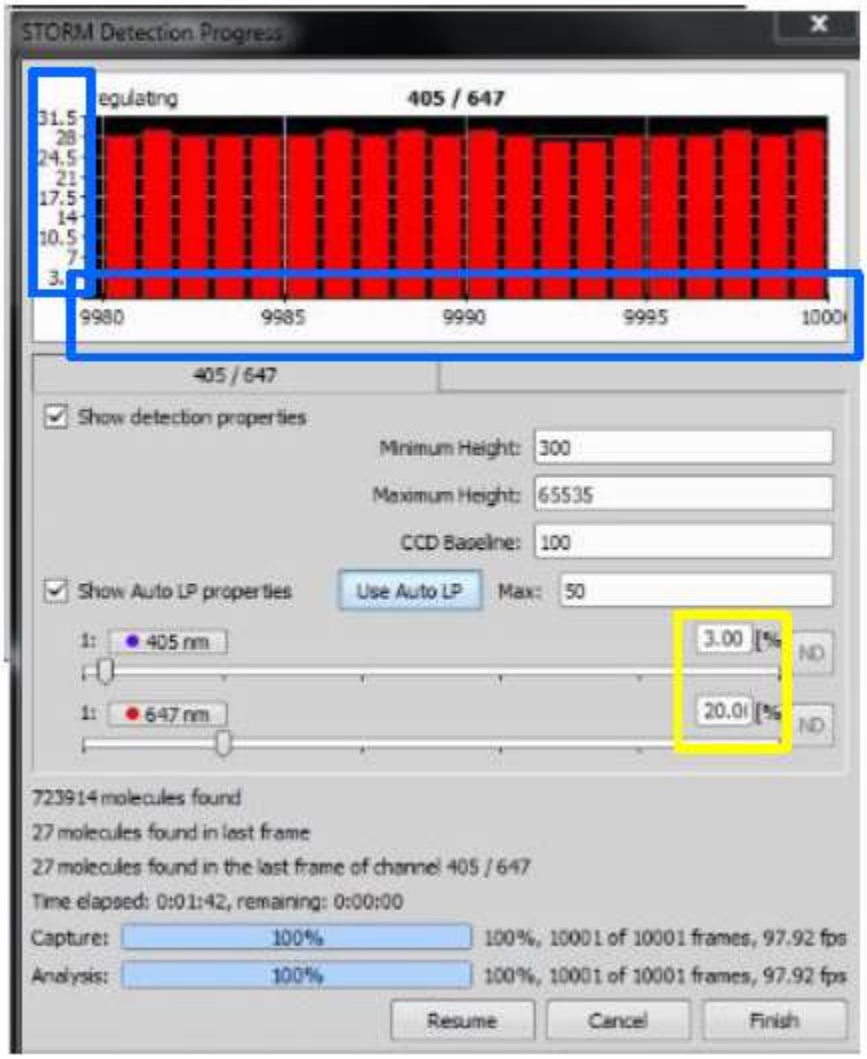
2) Click the button to plot the cross mark.

3) If you turn on “filters”, each filters to display the molecules works. You can set the range of position, intensity and size.

4) If you turn on “Time grouping”, frames are limited to display the molecules. If turn off, all frames are displayed.

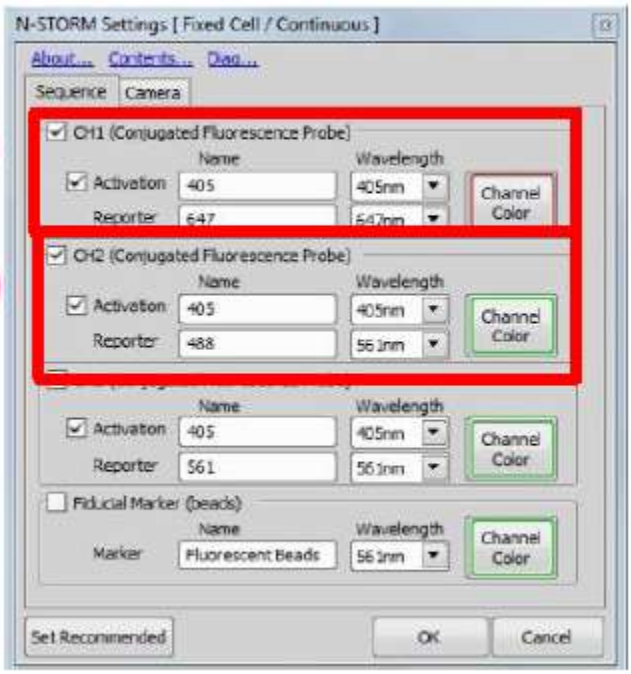


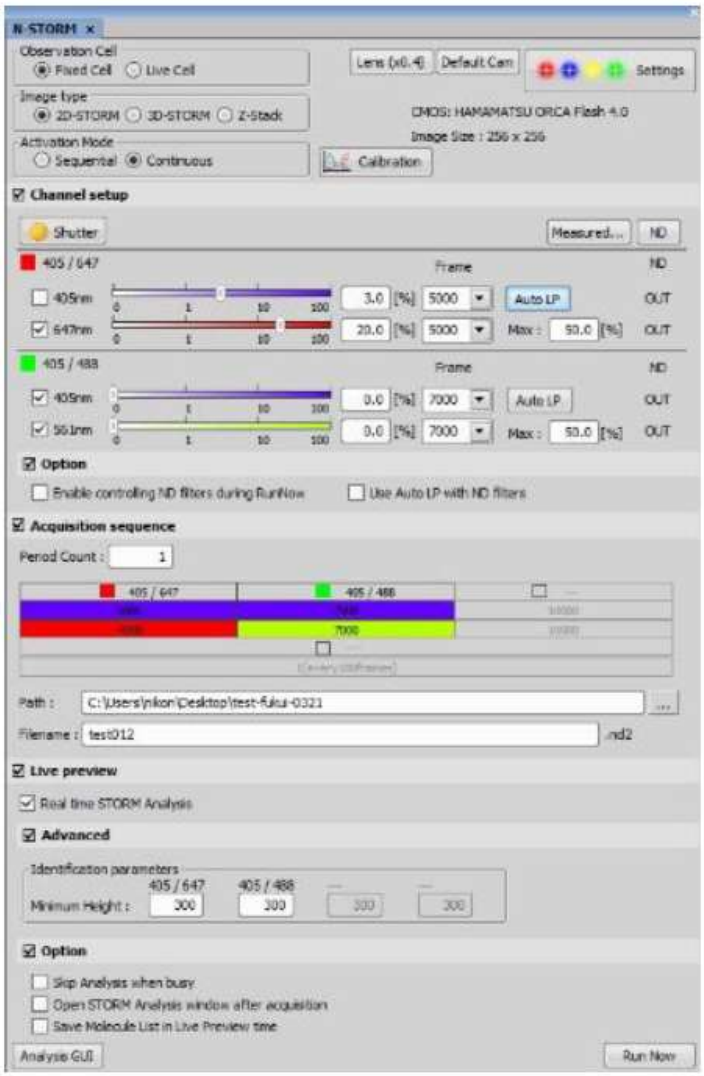
The progress window opens after acquisition starts. You can change the scale size using the mouse wheel by putting the pointer on the blue box. Laser power values in the yellow box are the same as setting values in N-STORM window.



Two Colour Acquisition

1) Turn on the CH1 and CH2 in the N-STORM settings window, then set the channel name and wavelength.
Now that N-STORM window changes shown as in the right, you can acquire the two color STORM images.





3D STORM Acquisition

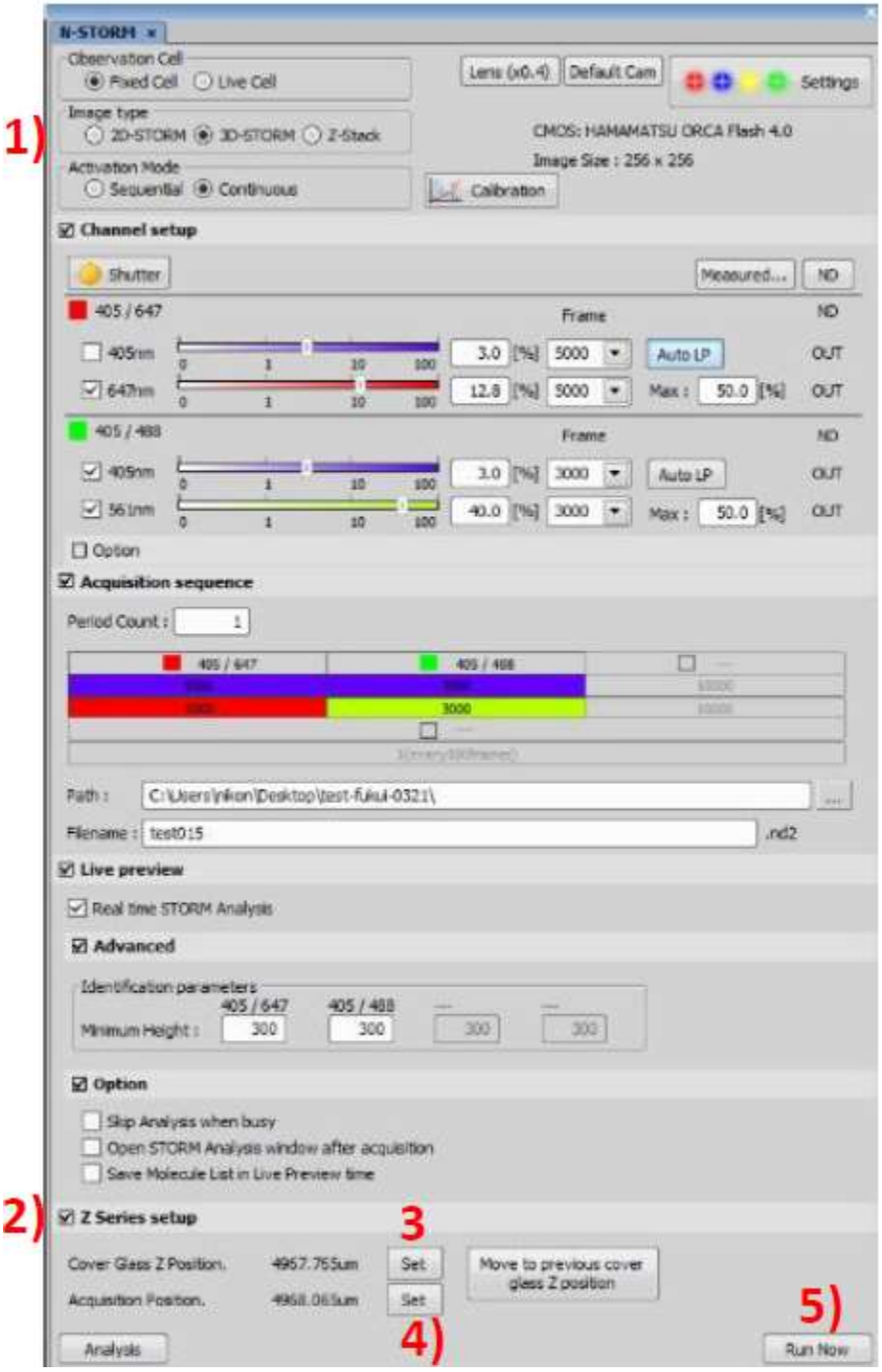
1) Select the “3D-STORM” in “Image Type”.

2) Turn on “Z Series setup”.

3) Focus on the surface of coverslip, click the upper “Set” button .

4) Focus on the sample, click the lower “Set” button.

5) After other settings, click the “Run now” button to start the acquisition.



Acquisition setting for capturing a Z stack

1) Select the “Z-Stack” in “Image Type”.

2) Turn on “Z Series setup”.

3) Focus on the surface of coverslip, click the top “Set” button .

4)Focus on the bottom of the sample, click the upper “Set” button. Adjust the laser beam angle for excitation, click the upper “Set” button.

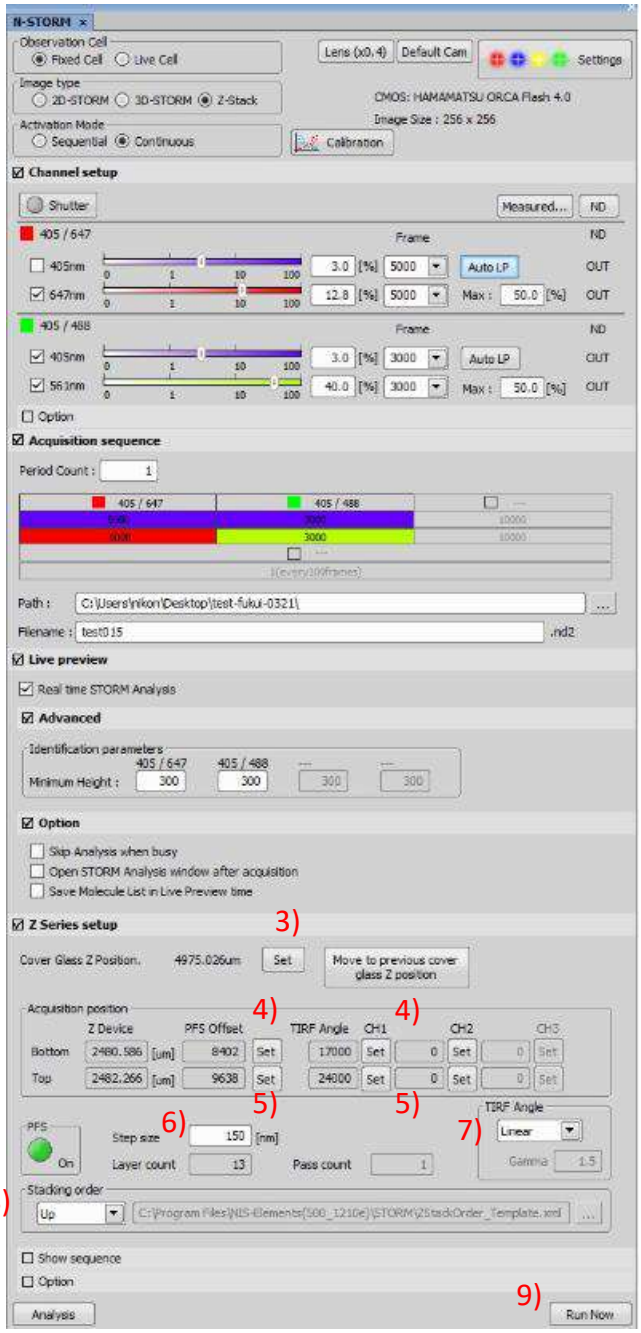
5) Focus on the top of the sample, click the lower “Set” button. Adjust the laser beam angle for excitation, click the lower “Set” button.

6) Set the “Step size”.

7) Set the “TIRF angle”.

8) Set the “Stacking order”.

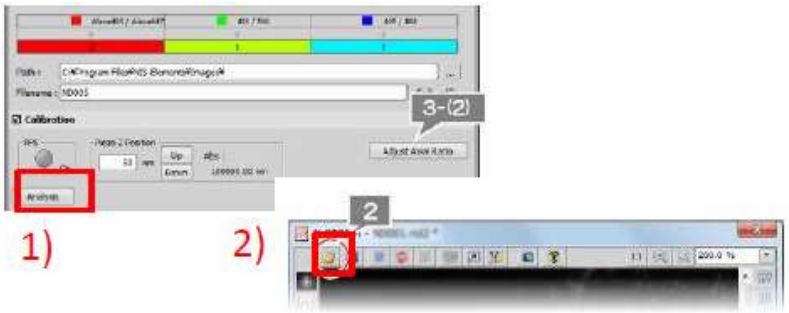
9) After other settings are done, click the “Run now” button to start the acquisition.



STORM Analysis

1). Load STORM image

1) Click [Analysis] to display the N-STORM analysis window.
2) Click [File Open] to open the image file (ND2 file) .

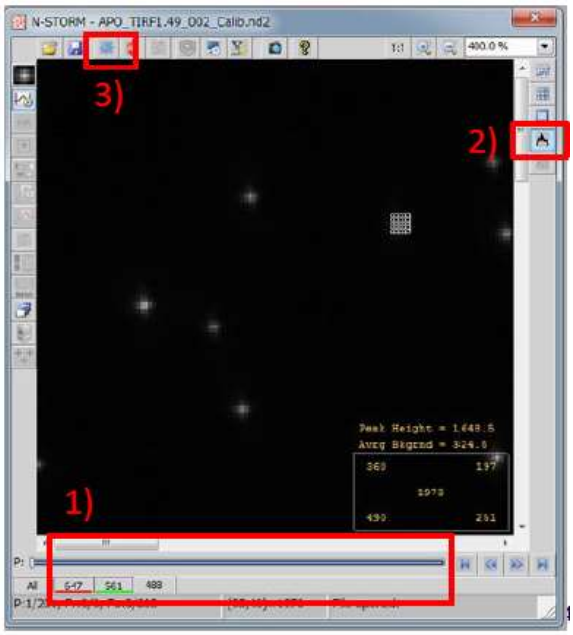


2. Setting for molecule analysis

1) Select the tab for each wavelength, and display the last image of the acquired image data.

2) Click [Use Peak Statistics] to display a 5 x 5 square box. Align the center of the box with the darkest of the bright spots and read the [Peak Height] value.

3) Click [identification Setting] to display the dialog box.



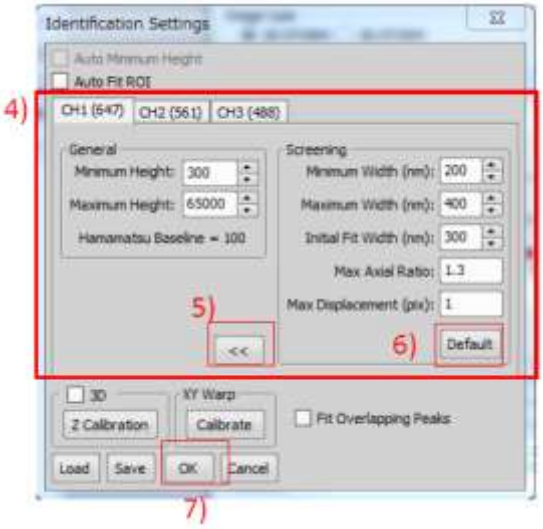
4) Set as follows for each channel in the dialog box.
Minimum Height: 2) value
Maximum Height: sCMOS 65000, EMCCD 16000 Auto Fit ROI: Off

5) Click [>>]

6) Click [Default] to Set all parameter as default

- Minimum Width (nm): 200
- Maximum Width (nm): For 2D-STORM = 400 For 3D-STORM = 700
- Initial Fit Width (nm): 300
- Max Axial Ratio: For 2D-STORM = 1.3 For 3D-STORM = 2.5
- Max Displacement (pix): 1

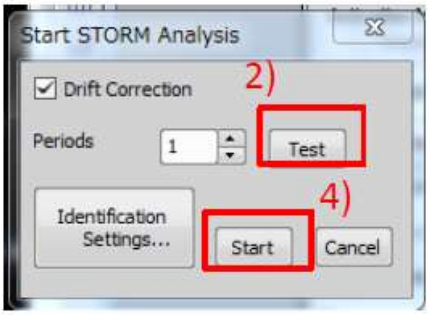
7) Click [OK].



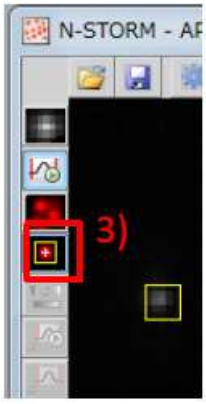
3. Molecule analysis

1) Click [Start STORM Analysis]

2) Run test analysis with [Test]

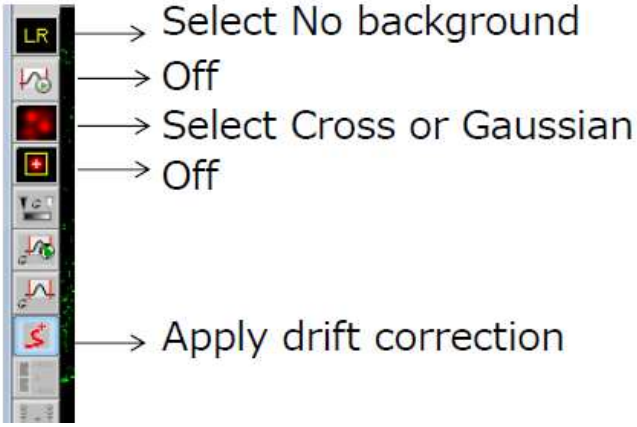


3) Turn [Mark molecules identified in current frame] on / off and confirm that the bright spots are correctly recognized.



4) Start analysis with [Start]

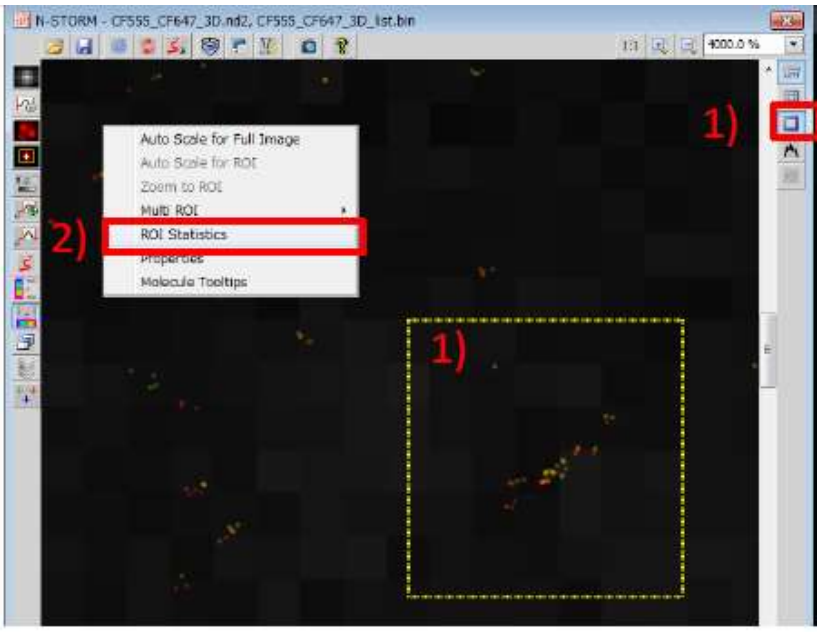
5) A super-resolution image is constructed and displayed



Quantitative Analysis

1) Select ROI

2) Display ROI Statistics dialog select [ROI Statistics] from the menu displayed by right-clicking outside the ROI frame on the N-STORM analysis window, the ROI Statistics dialog will be displayed.



3) Analysis results are displayed

3-1) Localization Accuracy Detection Bright spot

3-2) Photon Count Distribution

3-3) Ripley's K Function Next page for more detail