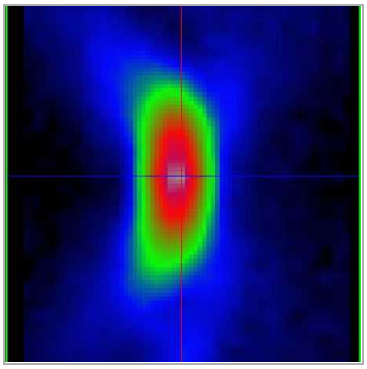

FEB 14, 2023

OPEN ACCESS

Protocol Citation: Rafael Camacho 2023. PSF measurements for ELYRA 7. **protocols.io** https://protocols.io/view/psf-measurements-for-elyra-7-cm8su9we

Protocol status: Working
We use this protocol and it's working

Created: Jan 24, 2023

Last Modified: Feb 14, 2023

PROTOCOL integer ID: 75762

Keywords: PSF, ELYRA 7

PSF measurements for ELYRA 7

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DISCLAIMER

ABSTRACT

Protocol for measuring PSFs at the ELYRA 7. This protocol was developed to follow guidelines from ZEISS application specialists. It is therefore, based on the tools and software provided by ZEISS.

As **sample** you can use the fluorescent beads provided by ZEISS which size is below the diffraction limit.

Equipment	
ELYRA 7	NAME
Microscope	TYPE
ELYRA	BRAND
431014-9903-000	SKU

Software	
ZEN black	NAME
ZEISS	DEVELOPER

BEFORE START INSTRUCTIONS

Make sure to read all steps of each section before you try to implement them.

Warming up the microscope

1 Turn on the ELYRA 7

1.1 Turn on **Main Switch**

1.2 Turn on **Components**

1.3 Turn on Microscope Computer

> **Note**
>
> In the ELYRA 7 if the microscope computer is not on, then the microscope's touch screen will not light up.

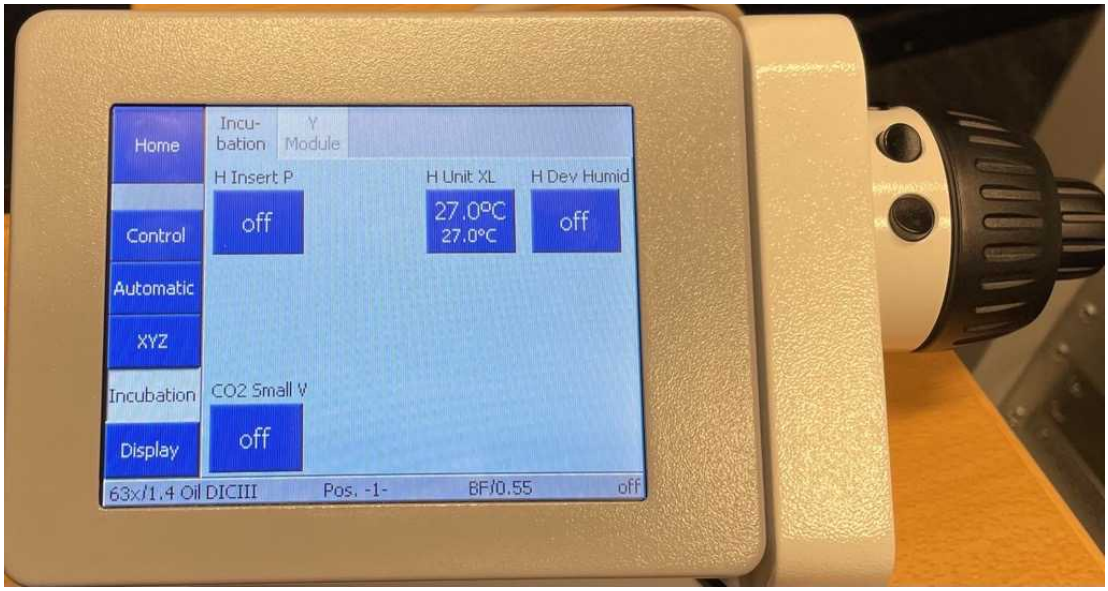
1.4 Open **ZEN black**, and choose *start system*

2 Let the microscope warmup overnight.
During this process make sure to leave the **sample**, and the **immersion oil** inside the chamber, so they also reach the desired temperature.

3 Temperature control for increased stability.

3.1 Check the temperature of the imaging chamber (incubation XL unit).
In our case it stabilizes at around 26-27 degrees when the microscope is on.

3.2 Taking into account the result of step 3.1, stabilize the temperature of the microscope. In our case we use 27 degrees. This can be done via:
1) **Touch screen** [Home -> Microscope -> Incubation -> H Unit XL -> Set Temperature -> Set On -> Click ok]



2) **ZEN black** [Locate Tab -> Incubation -> Temperature -> H unit XL checkbox]

Align the stage

4

The stage must be perpendicular to the microscope to avoid imaging artifacts.
Please follow the protocol:

Protocol

NAME

Insert Alignment ELYRA7

CREATED BY

Rafael Camacho

PREVIEW

Find focus

5 Engage the objective you want to use. In our case Plan-Apochromat 63X / 1.4 Oil

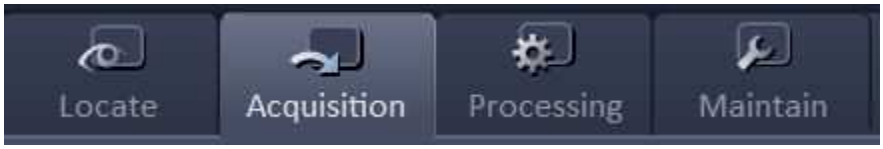
6 If necessary put a small drop of immersion fluid on the objective

7 Place the sample on the holder

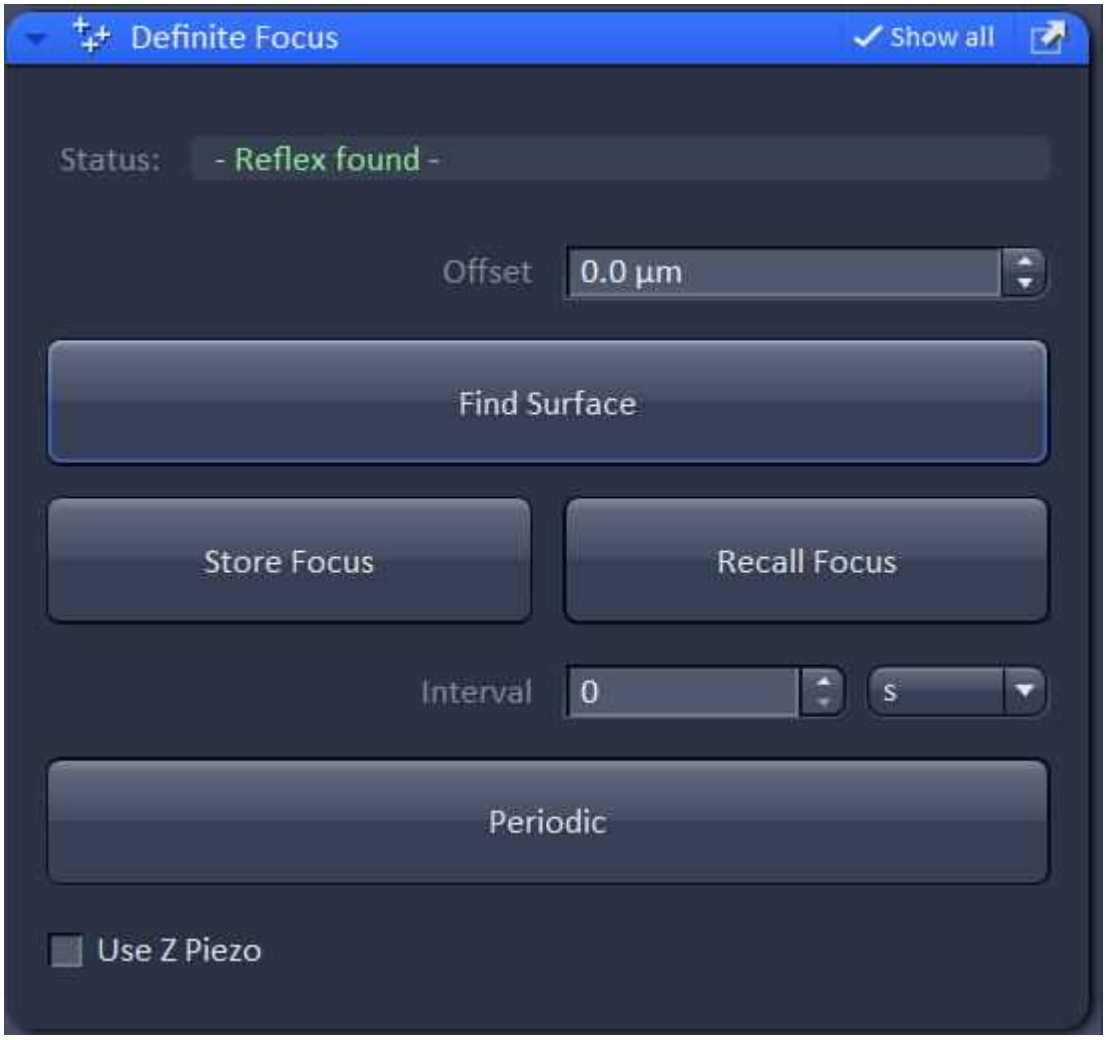
8 Manually move the objective up until it touches the immersion fluid

9 You can approximate focus by using the **Definite Focus**

9.1 Zen black -> Acquisition Tab

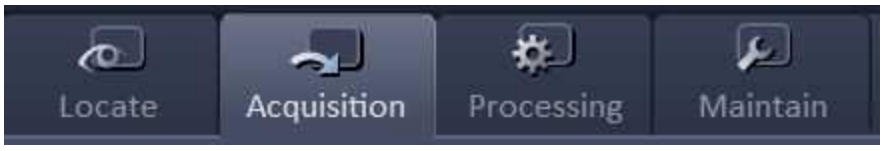


9.2 Acquisition Parameter -> Definite Focus Menu -> Find Surface

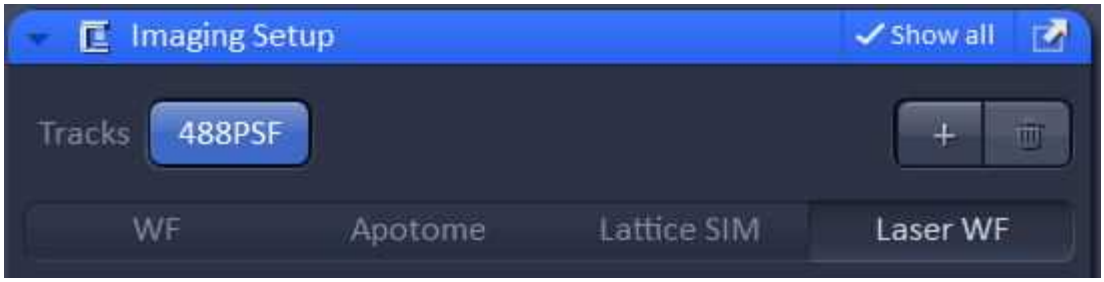


10 Set imaging parameters, here we use Laser WF mode. Below an example for the 63X objective:

10.1 Zen black -> Acquisition Tab



10.2 Select the Laser WF mode by
Setup Manager -> Imaging Setup ->Laser WF



In following sub-steps you will find detailed information about our experiment configuration. We recommend that you store these settings via the experiment manager. In our case we stored this as "488PSF"

10.3 Laser WF mode settings:

- 63x / 1.4 oil objective
- Dichroic: BP 495-550 + BP 570-620
- Emission beam splitter (Duolink): SBS 490-560 + LP 640
- Active Camera TV1

10.4 Channel Settings

Acquisition Parameter -> Channels:

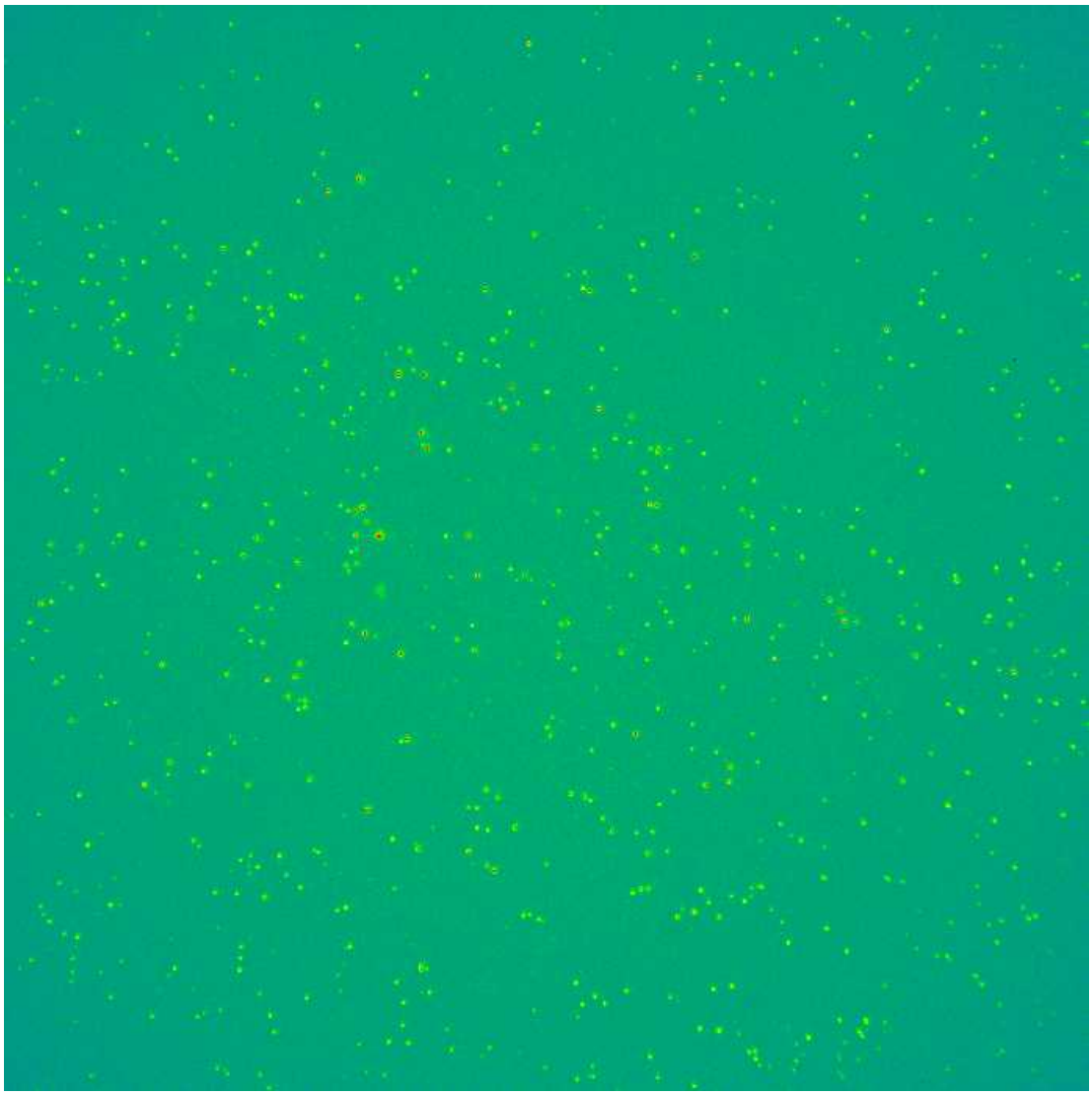
- 488 nm excitation, 4%
- Illumination mdoe: EPI
- Exposure time 100-300 ms

11 Go to live mode and fine adjust for focus (for reduced field of view, use **Continious** instead)

Live button

Live active

Expected result



Drift stability test

12

Note

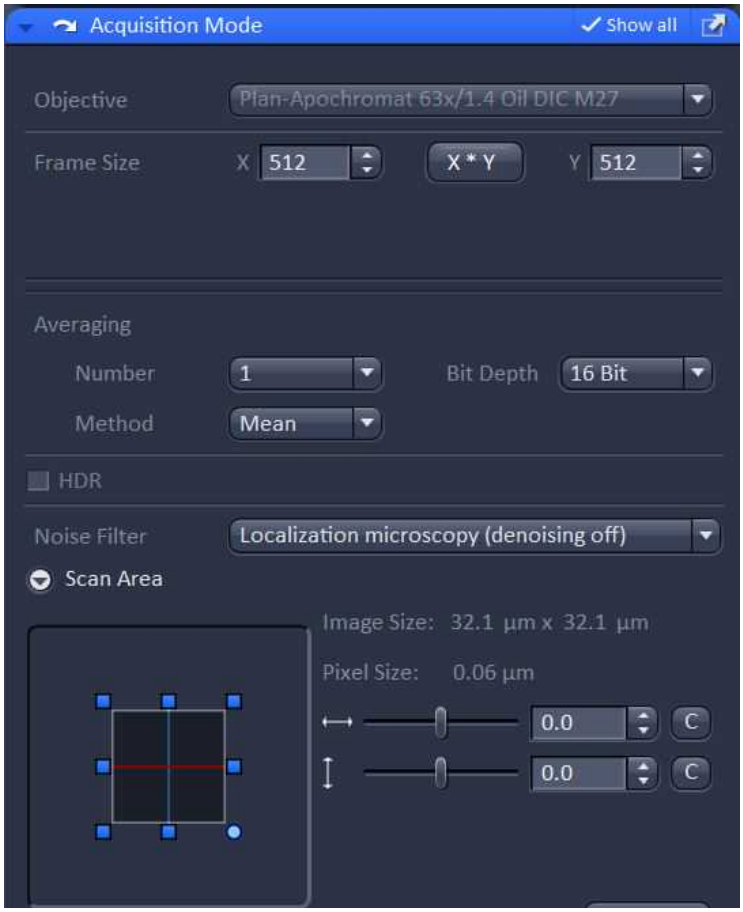
PSF measurements take time. Thus we want to make sure that there is no sample drift before starting. Drift will induce elongations of the output PSF.

Reduce your field of view to about 30 micron^2 This can be done by cropping the camera chip

12.1 Acquisition Parameter -> Acquisition Mode:

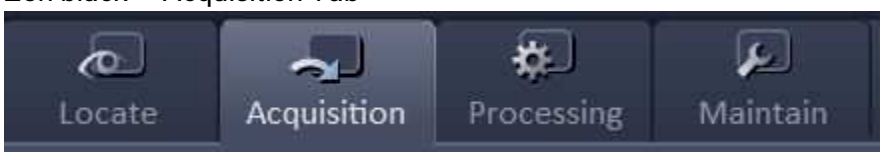
- Frame size e.g. 512 x 512

Make sure images are 16 bits for better performance during analysis

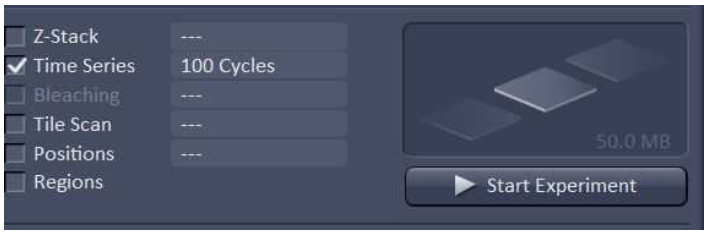


13 Generate a Time Series experiment, with a length of 20 minutes and 0.1 fps

13.1 Zen black -> Acquisition Tab

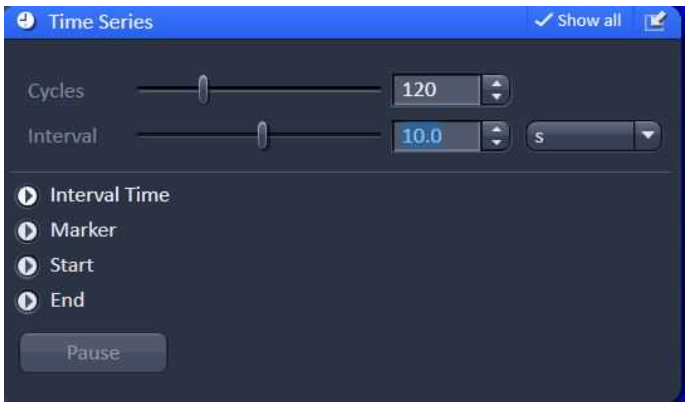


13.2 Activate Time series in Experiment manager



13.3 Multidimensional Aquisition -> Time Series

- Cycles 120
- Interval 10.0 s



13.4 Find focus: go to step #5 if you need help

13.5 Start Experiment via Experiment Manager -> Start experiment

14 Review the stability of the drift by looking at your time series. The beads image should not move or get out of focus.

Note

It can take up to 20 minutes for a sample to stabilize.

Expected result

Sample should not drift within the time frame of your experiments (usually a few minutes).

Bleaching test

15

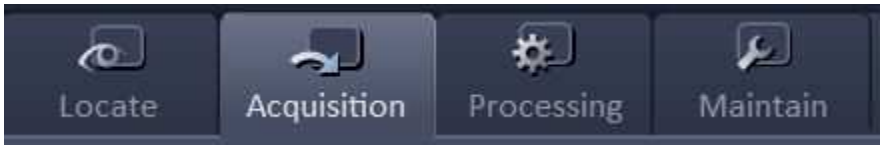
> **Note**
>
> During PSF measurements we acquire a Z-stack. Therefore, the sample should not significantly bleach during a time series experiment of the same length as your Z-stack. If not your PSF shape will be deformed due to bleaching.

Reduce your field of view to about 30 micron^2 This can be done by cropping the camera chip.

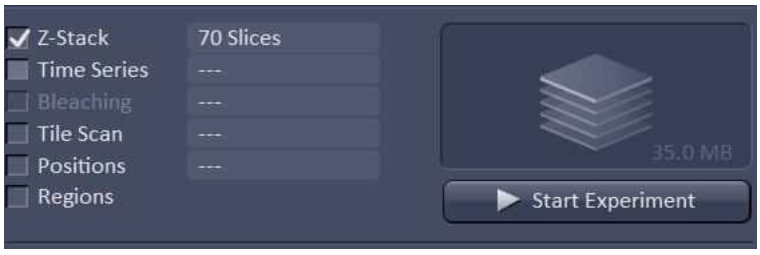
go to step #12.1 for details

16 Check the size of your desired z-stack. Example for 63x 1.4 oil objective:

16.1 Zen black -> Acquisition Tab

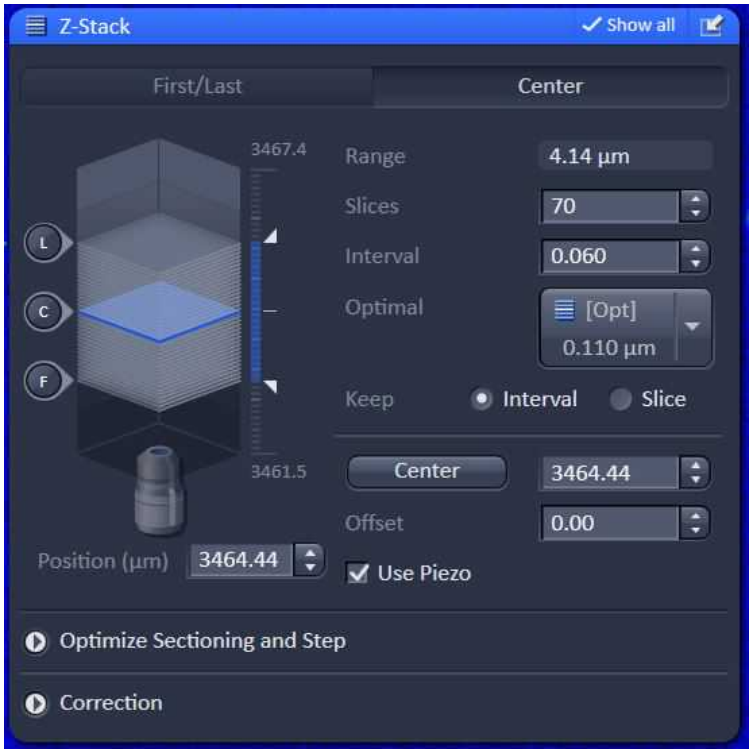


16.2 Experiment Manager -> Z-stack



16.3 It is important that your Z-stack has a resolution of approximately 1/2 times the optimal step.
Multidimensional Acquisition -> Z-stack

- Keep interval [active]
- Interval 0.06
- Set a number of slices that gives you a range >= 4 microns
- Use Piezo [active]

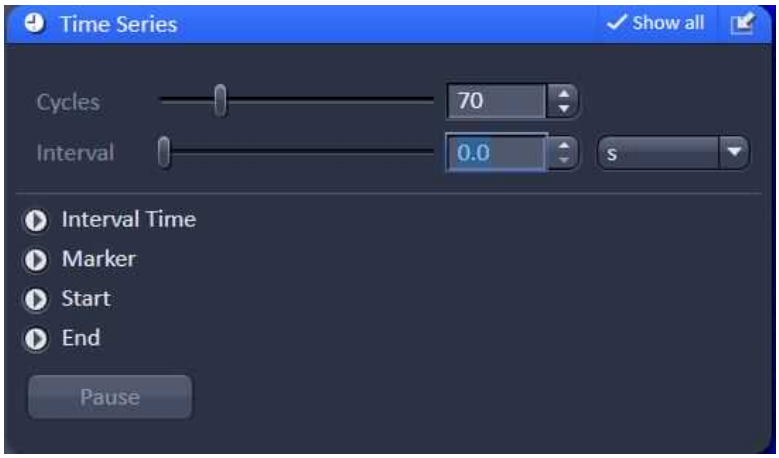


16.4 Take note of the number of **Slices**, in our example 70, and deactivate the z-stack option via Experiment Manager -> Z-stack

17 Acquire a time series with a number of frames equal to the number of slices determined in go to step #16.4 and no time interval (continious imaging)

For a time series example go to step #13.1

Example:

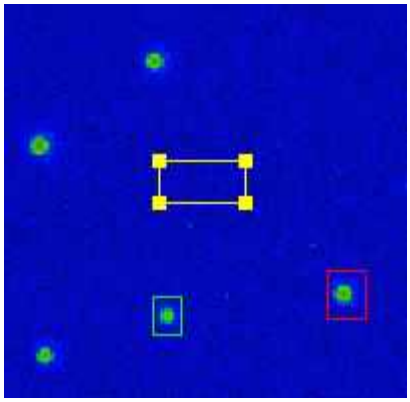


18 Review the bleaching of the beads. Below an example using **Mean ROI** tool in **ZEN black**:

18.1 ZEN Image Viewer -> Mean ROI

18.2 Draw a rectangular ROI around some beads and background area

Drawing tools

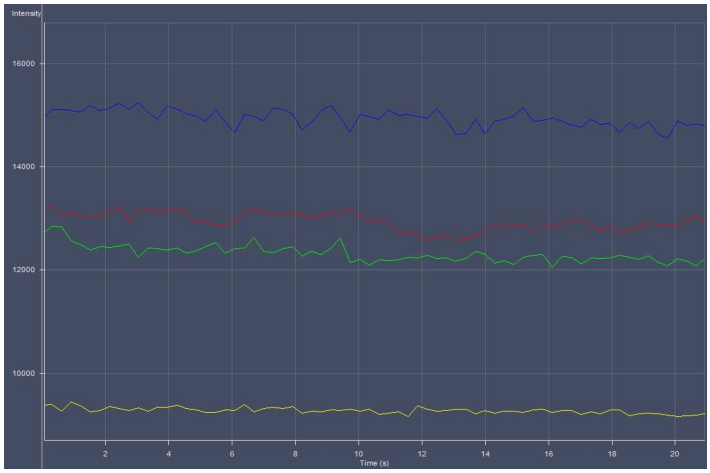


Example of selection

18.3 Check that you have no significant bleaching of the beads over the time frame of your experiment

Expected result

Here an example:



Measuring the PSF

19 Please note that there are 3 configurations for which we want to measure the PSF

1) Laser WF Tubelens/Optobar 1.0 - Most relevant for SMLM
2) Laser WF Tubelens/Optobar 1.6 - Used as a comparison to the SIM PSF
3) Lattice SIM Tubelens/Optobar 1.6 - Used for Lattice SIM

Step 19 includes a Step case.

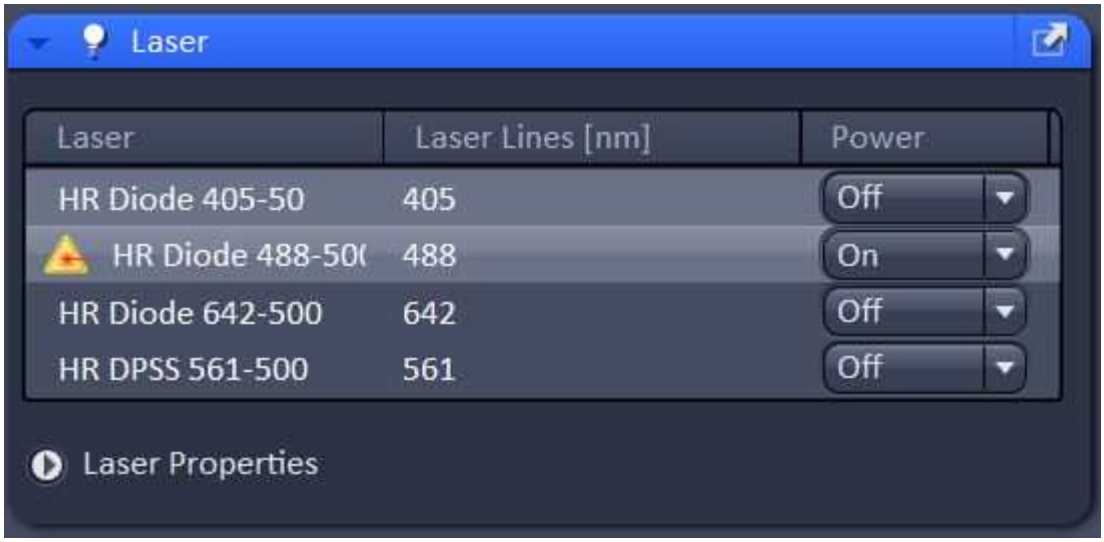
Laser WF 1.0 TL

Laser WF 1.6 TL

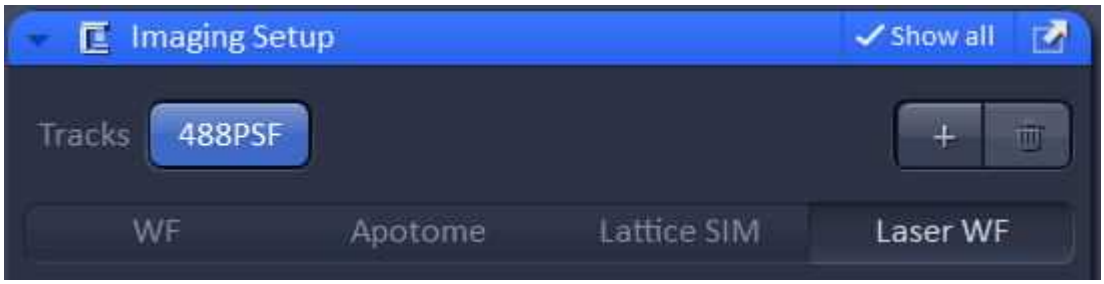
Lattice SIM 1.6 TL

Laser WF 1.0 TL

20 Setup Manager -> Laser
Activate the 488 laser



21 Imaging Setup -> Laser WF

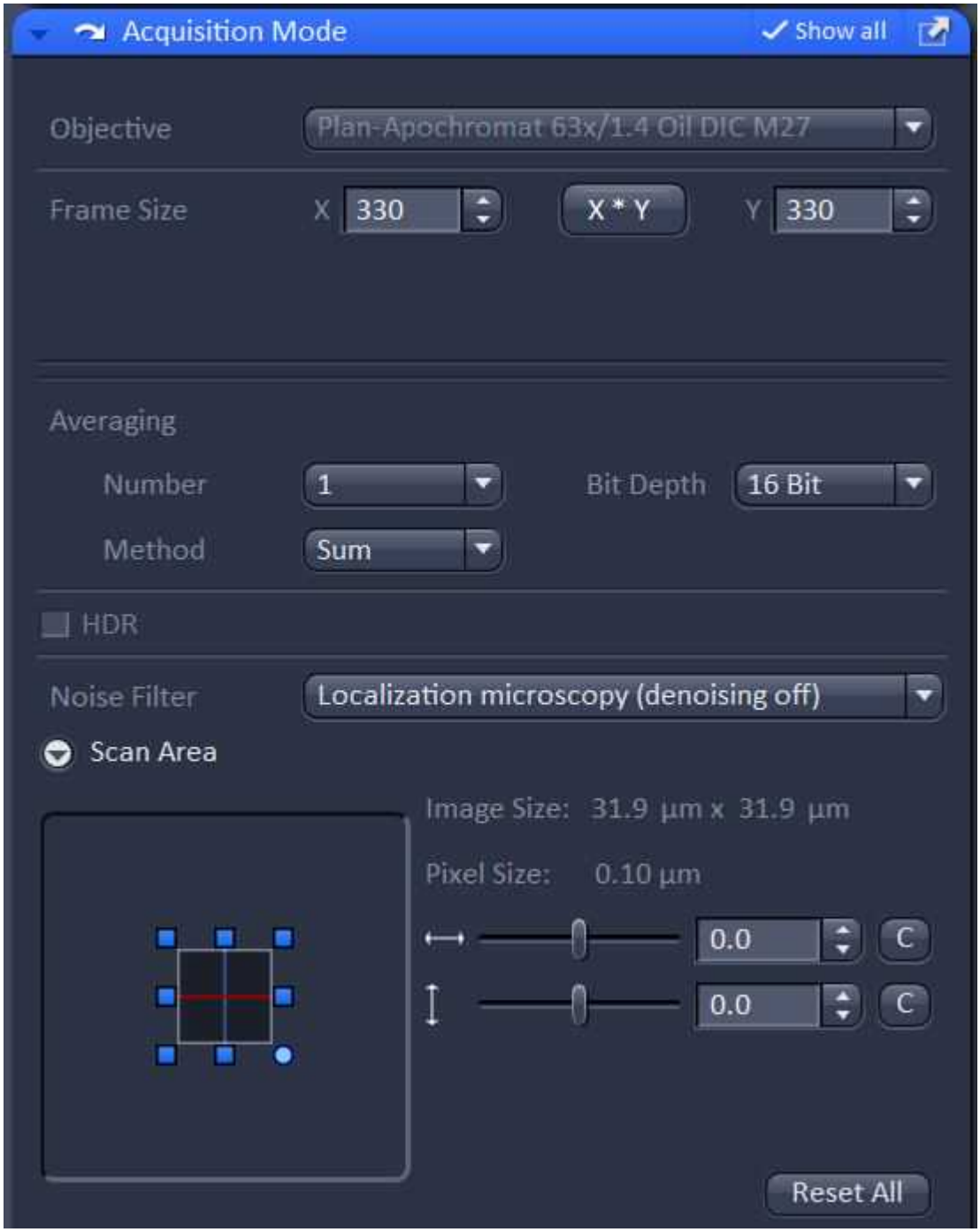


- Objective Plan-Apochromat 63x / 1.4 Oil
- Reflector revolver (dichroic filter): BP 495-550 + BP 570-620
- Tube lens (Optobar): 1x
- Dual Camera Beam Splitter: SBS BP 490-560 + LP 640
- Channel Camera: TV1

22 Acquisition Parameter -> Acquisition Mode

- Frame size: aim for an area of 32 micron^2 this translated to 330 x 330 pixels
- Bit Depth 16
- You might consider averaging or sum in some cases
- Noise filter: denoising off

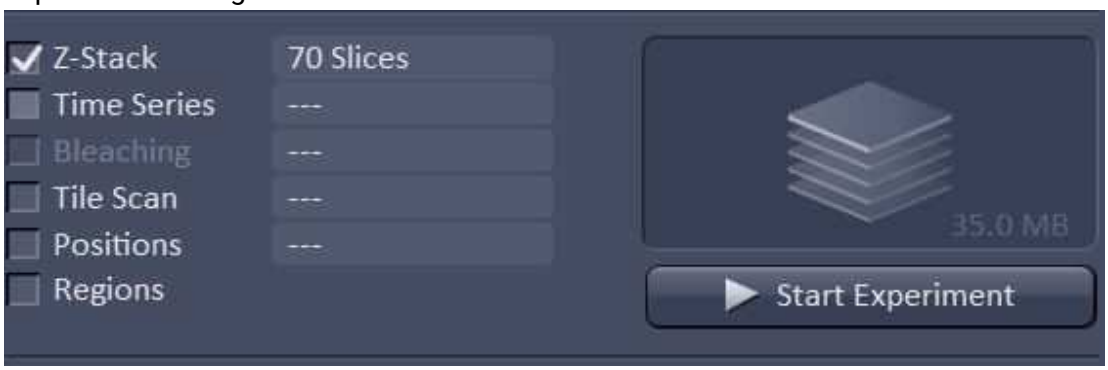
Note how the images are taken in the centre of the field of view


Acquisition Mode
Show all
Objective
Plan-Apochromat 63x/1.4 Oil DIC M27
Frame Size
X
330
X * Y
Y
330
Averaging
Number
1
Bit Depth
16 Bit
Method
Sum
HDR
Noise Filter
Localization microscopy (denoising off)
Scan Area
Image Size: 31.9 µm x 31.9 µm
Pixel Size: 0.10 µm
0.0
C
0.0
C
Reset All

23

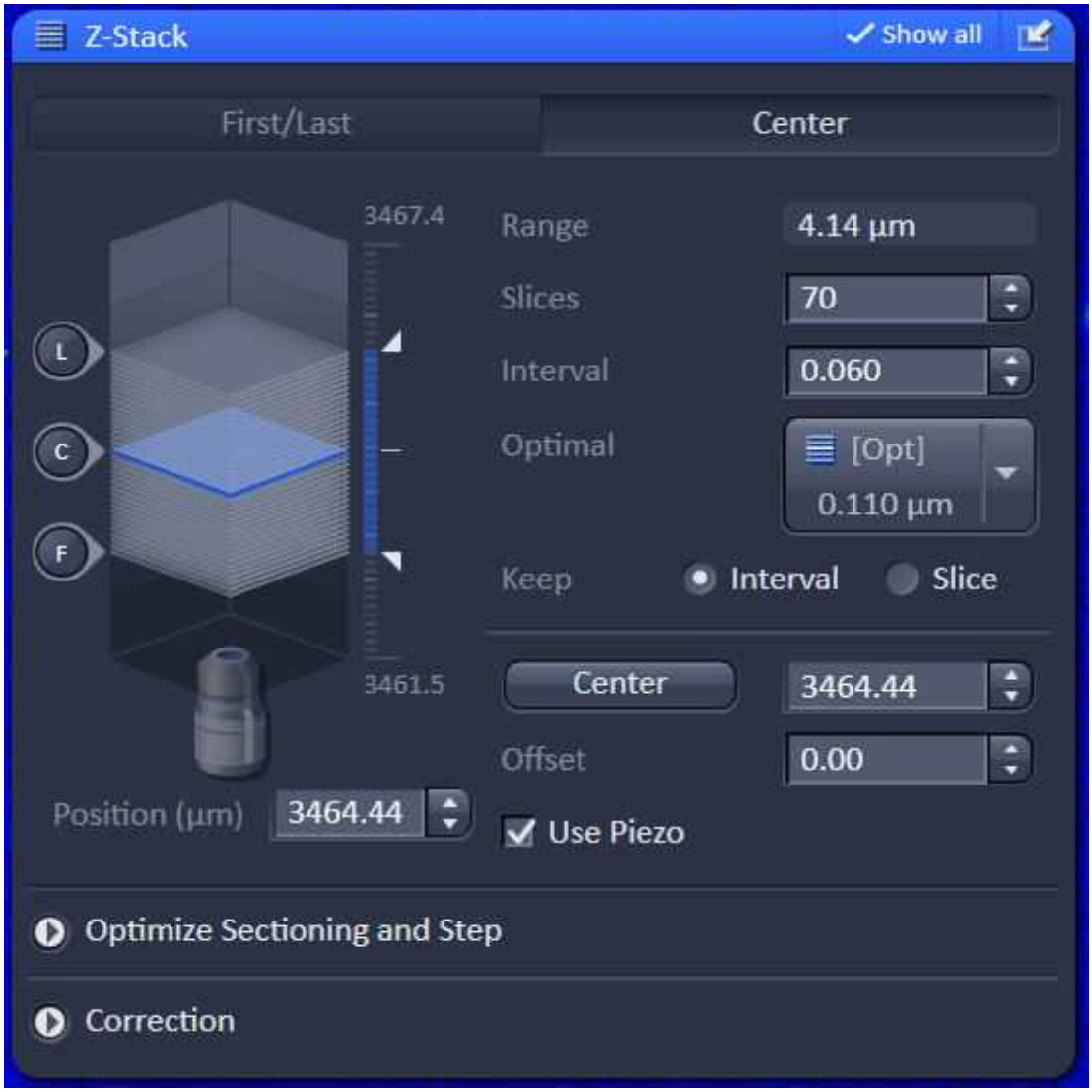
24 Setup the Z-stack

24.1 Experiment Manager -> Z-stack

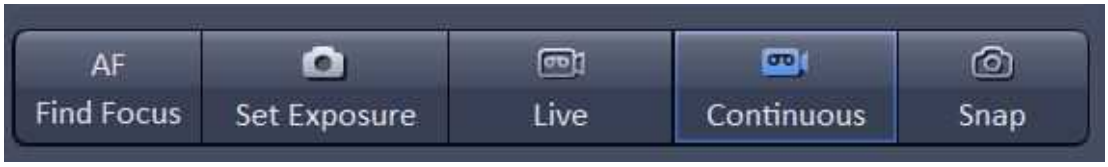


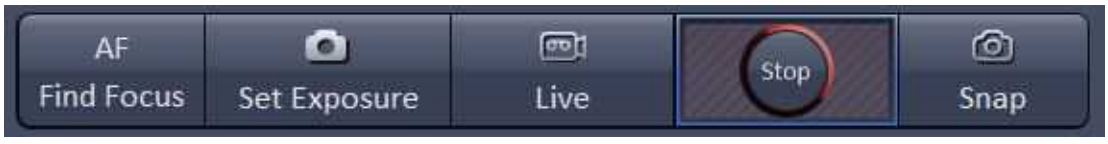
24.2 It is important that your Z-stack has a resolution of approximately 1/2 times the optimal step.
Multidimensional Acquisiton -> Z-stack

- Keep interval [active]
- Interval 0.06
- Set a number of slices that gives you a range >= 4 microns
- Use Piezo [active]

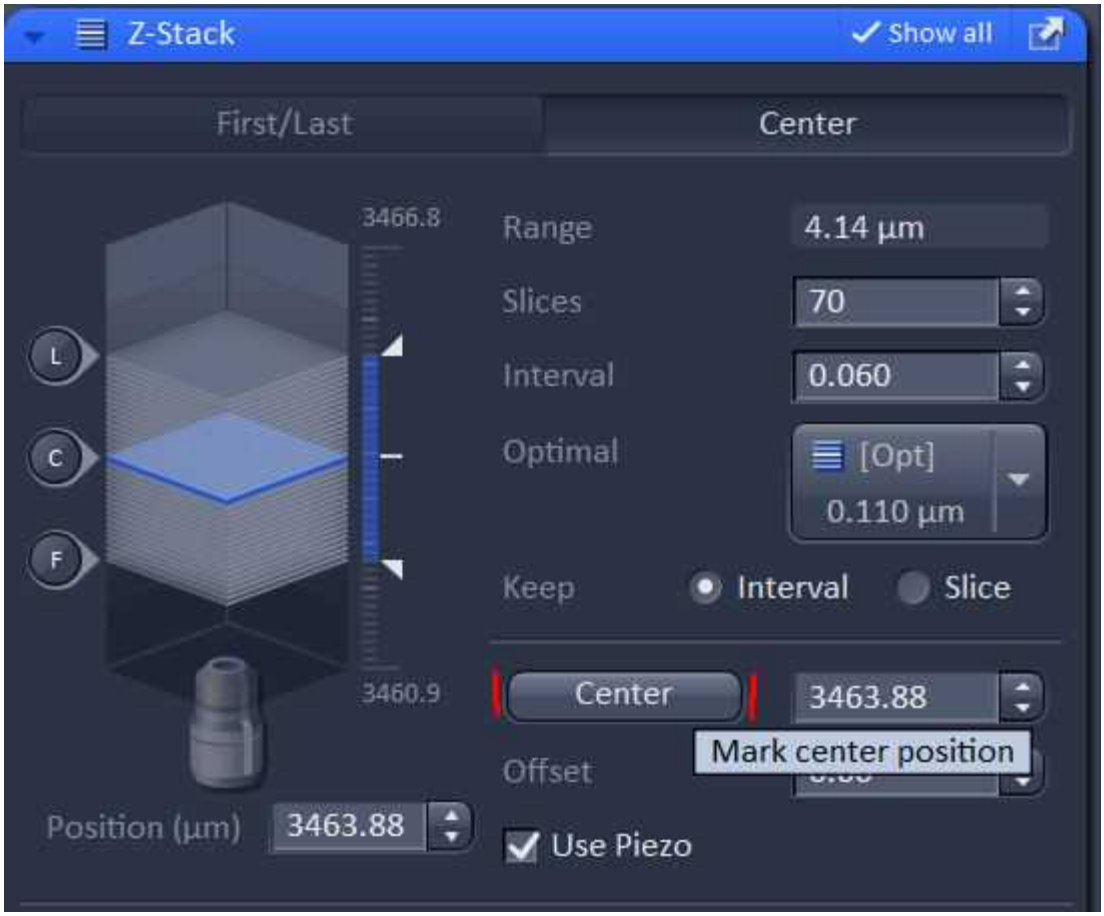


25 Go to Continuous mode and adjust focus





26 Multidimensional Acquisition -> Z-stack -> Center mode -> click on center



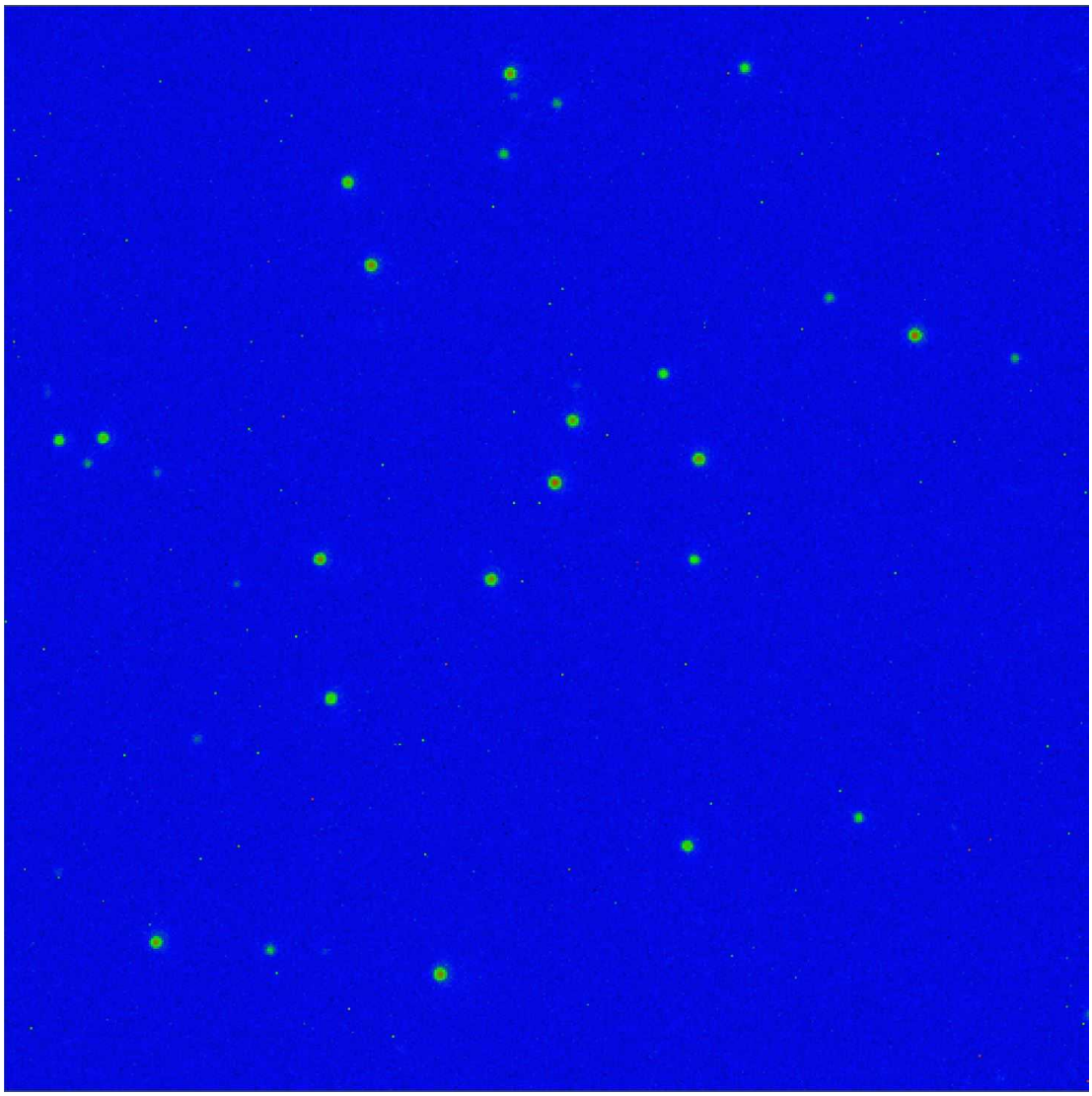
27 Start Experiment via Experiment Manager -> Start experiment

Analysis of PSF data - Widefield

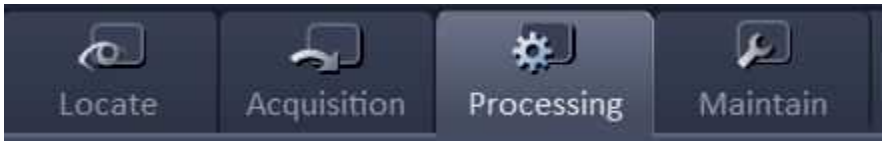
28 Read all steps of this section before you try to implement them

29 Open the PSF data (z-stack)
ZEN black -> File -> Open

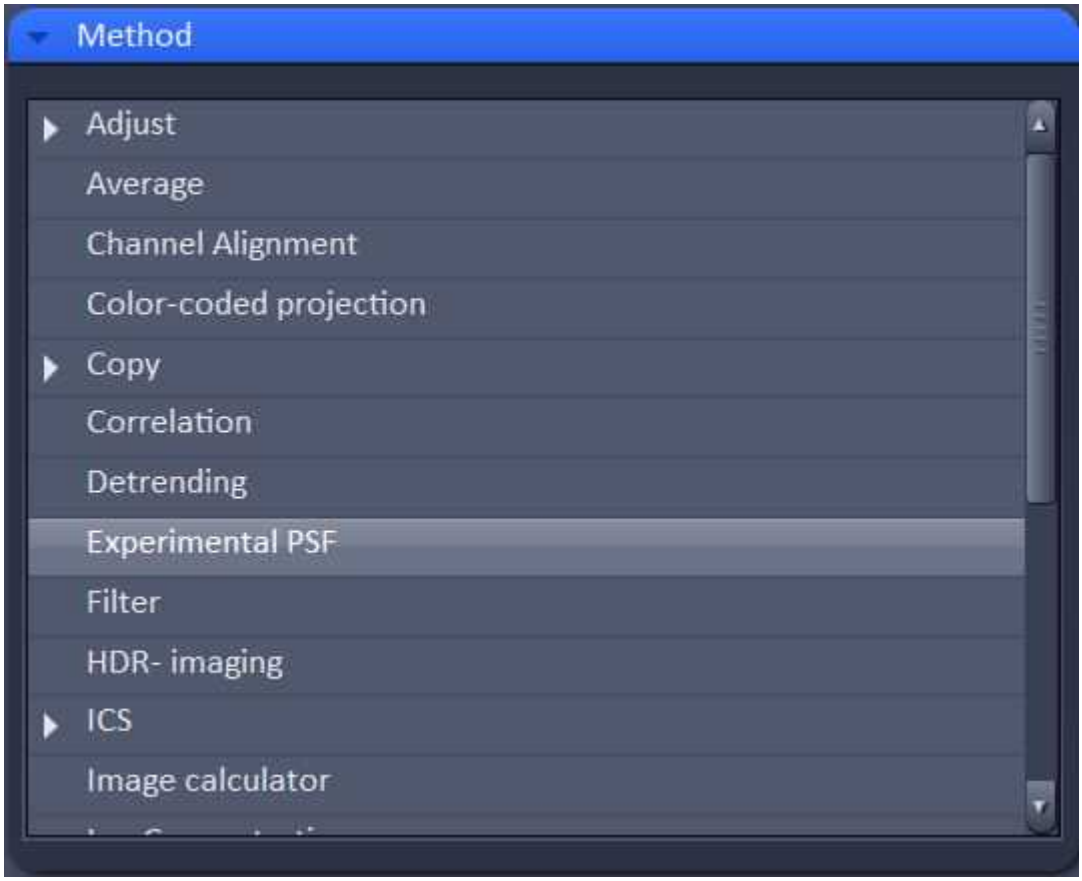
Expected result



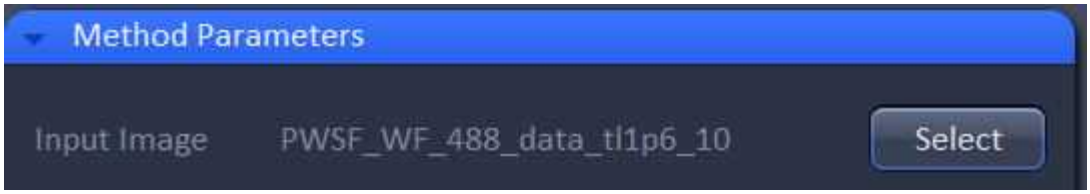
30 Go to the **Processing** tab



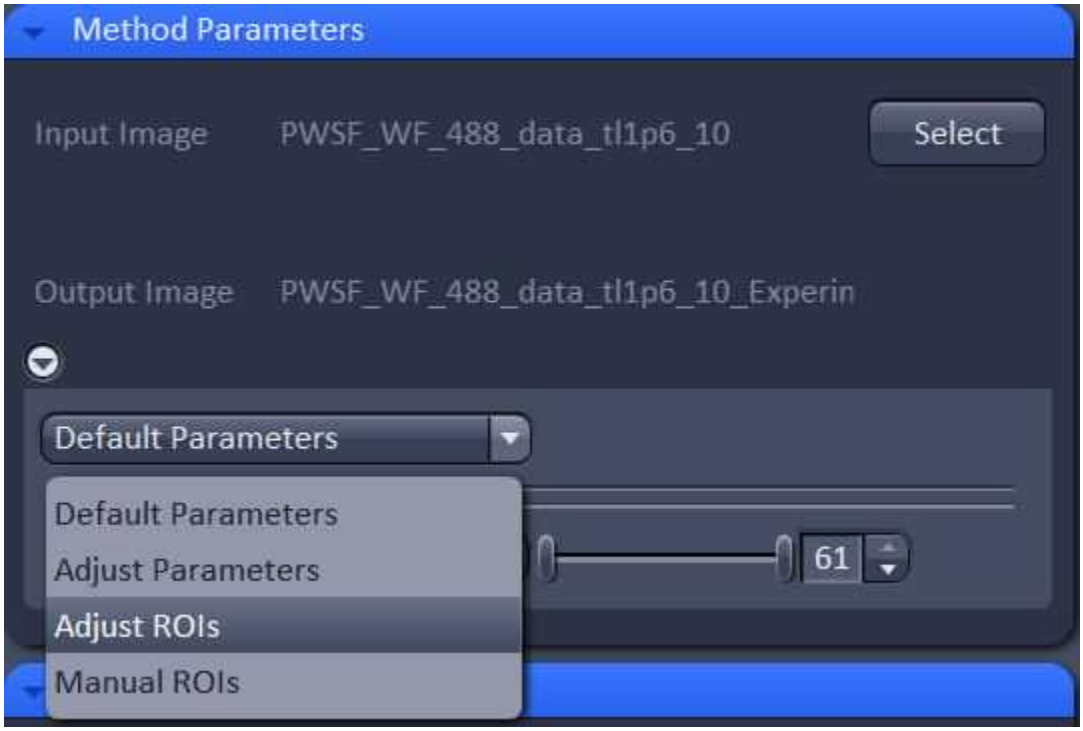
31 Method -> Experimental PSF



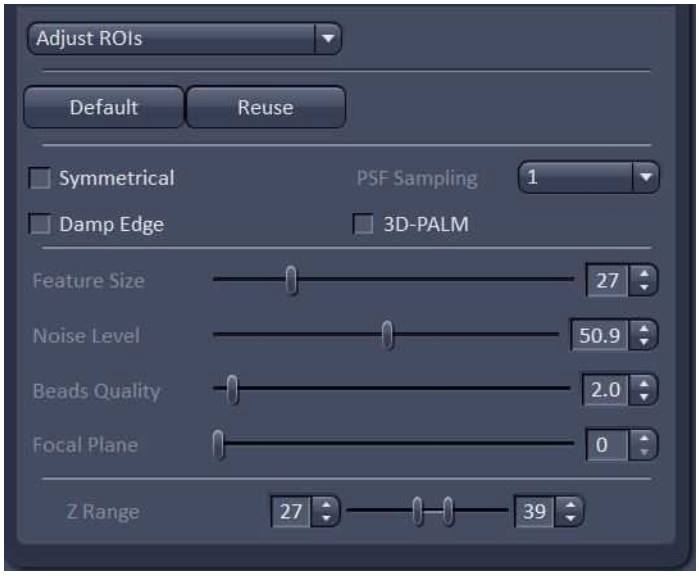
32 Method Parameters -> Select button



33 Method Paramters -> Change to Adjust ROIs



34 **Parameters:** there is no set of parameters that will fit all your experiments. Here we provide some information regarding the parameters. Please consider the numbers as a suggestion, or starting point, not as a fixed value that must be use for all cases.



34.1 Feature Size: 30

> **Note**
>
> From ZEN help: "sets the mask size that is used to cut out the bead and to create the Experimental PSF. The extensions are displayed in the Preview tab of the image container as circles."

Advice: Select a feature size as large as posible. The limitting factor will be the overlap between the beads in the image. We usually work with about 30 pixels for TL 1,6

34.2 Noise Level: 50

> Note
>
> From ZEN help: "sets the threshold of the intensity of the sub-resolution beads over background according to I – M > SD x NL (I = intensity of bead; M = mean intensity of image frame; SD = standard deviation of image frame; NL = noise level). Lowest number means that everything (even noise) is taken into account; highest number means only the brightest beads will be used."

Advice: It is a good idea to be stringent with this parameter (to use large values). The lower the value the more "initial" detections are considered. If the value is two low even noise spikes are considered as detected spots. We usually work with about 50.

34.3 Beads Quality: 2

> Note
>
> From ZEN help: "evaluates two characteristics, the intensity (first moment) and the size (second moment). These two moments are calculated for every localized bead. Then the beads are sorted according to these characteristics, resulting in two sorted lists of beads. The beads that are closer to the medians in both orderings are assumed to have higher quality. The Beads quality defines how far the beads characteristics (intensity and size) may be away from the median value. The smaller the threshold, the closer beads should be to the median."

Advice: We use small numbers (usually 1-3), however if the data set is clean we do not see much effect on this feature.

34.4 Z-range: Selects the number of slides from the z-stack that are used for spot detection.
Advice: Generally we set this to +-5 from the centre of the z-stack.

34.5 **Display:**

> Note
>
> From ZEN help: "All sub-resolution beads that will be taken into account will be circled by a white line. Those that do overlap with their circles or are considered unsuitable (beads beyond the quality thresholds) are outlined in red and will not be taken into account."

34.6 **PSF sampling:** usually 1 or 3

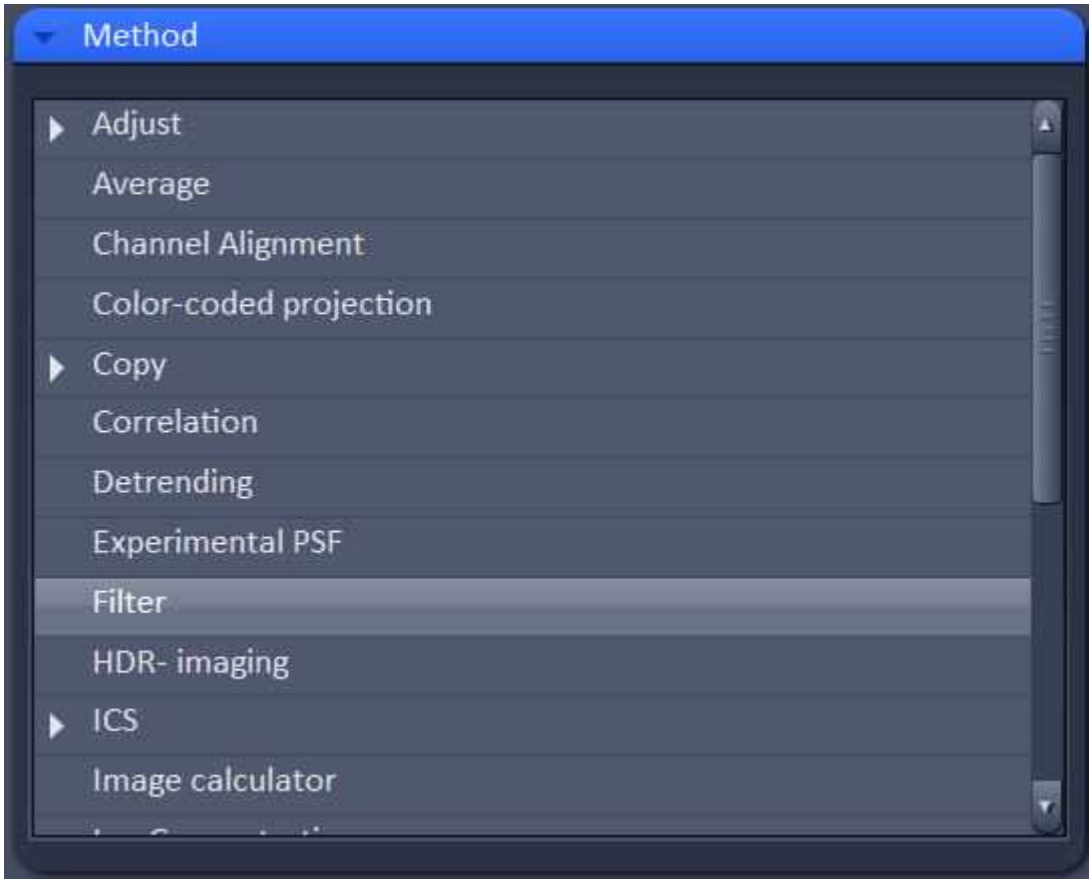
> Note
>
> From ZEN help: "The PSF sampling pull down menu allows you to interpolate the result to different degrees. Since the resulting PSF is an average of many PSFs corresponding to the localized beads and every bead is localized with the sub-pixel accuracy, one can obtain after averaging a PSF with higher resolution"

34.7 To apply the method and get the PSF click on apply:

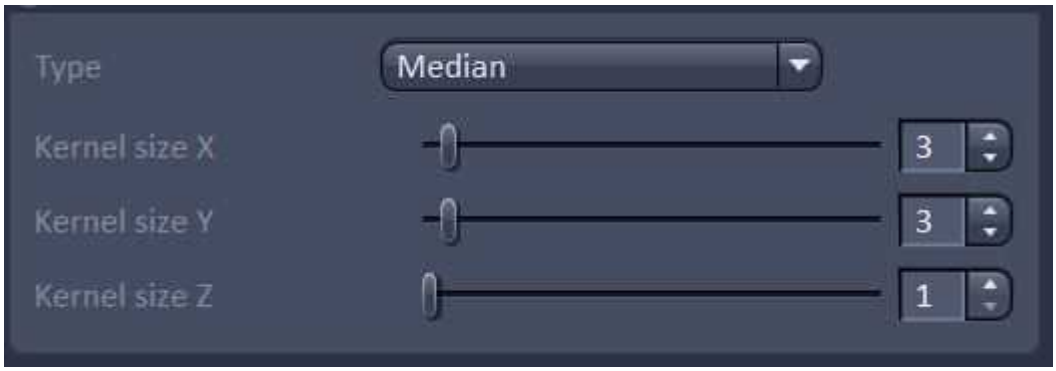
35 **How to overcome hot pixels in the PSF raw data** (slat pepper noise)

> Note
>
> **Strategy:** Removing hot pixels via median filter. Then use the filtered image to detect the spots of interest and save these selections. Finally load the selections manually for analysing the raw data to avoid the loss of resolution due to the filtering.

35.1 Processing tab -> Method -> Filter



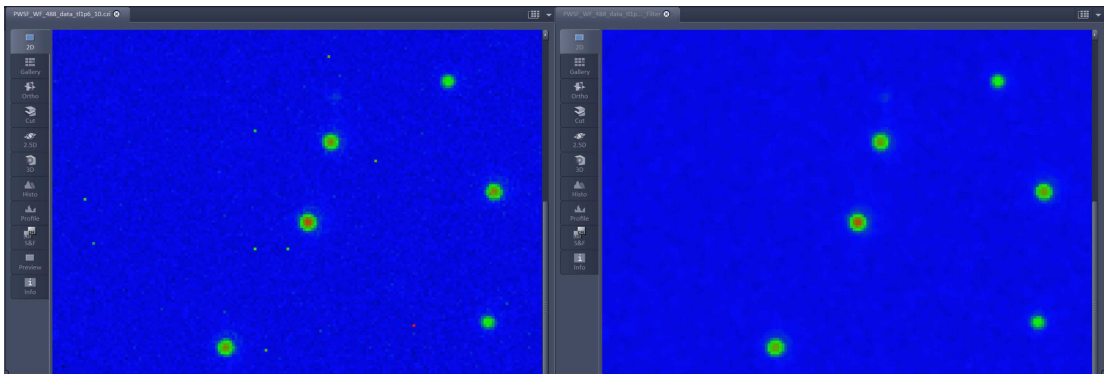
35.2 - Select **Input Image** (select button)
- Use filter **Type** Median
- Kernel size:
  Kernel size X: 3
  Kernel size Y: 3
  Kernel size Z: 1



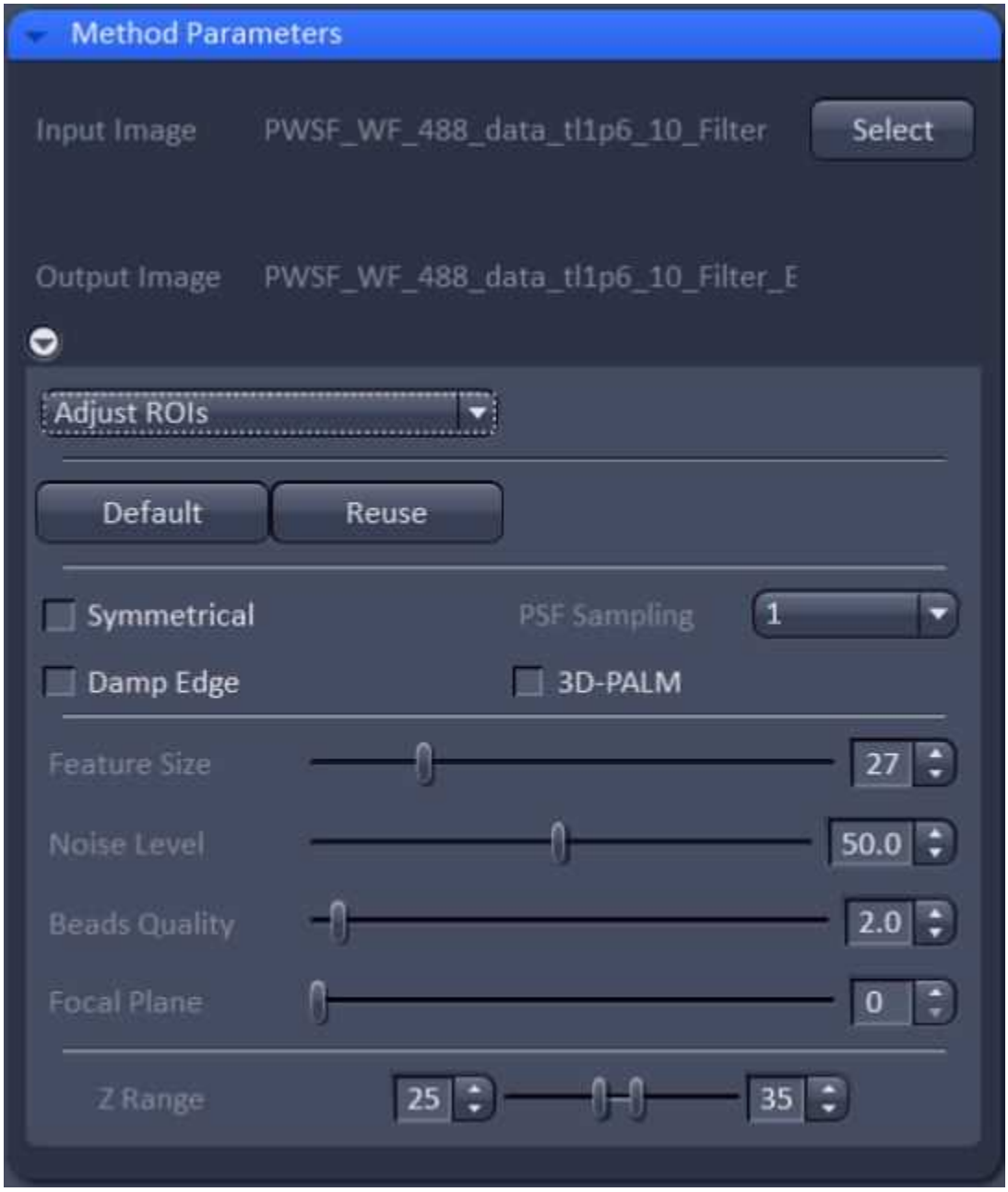
35.3 Click on **Apply**

This will create a new image document with the filtered result

Expected result



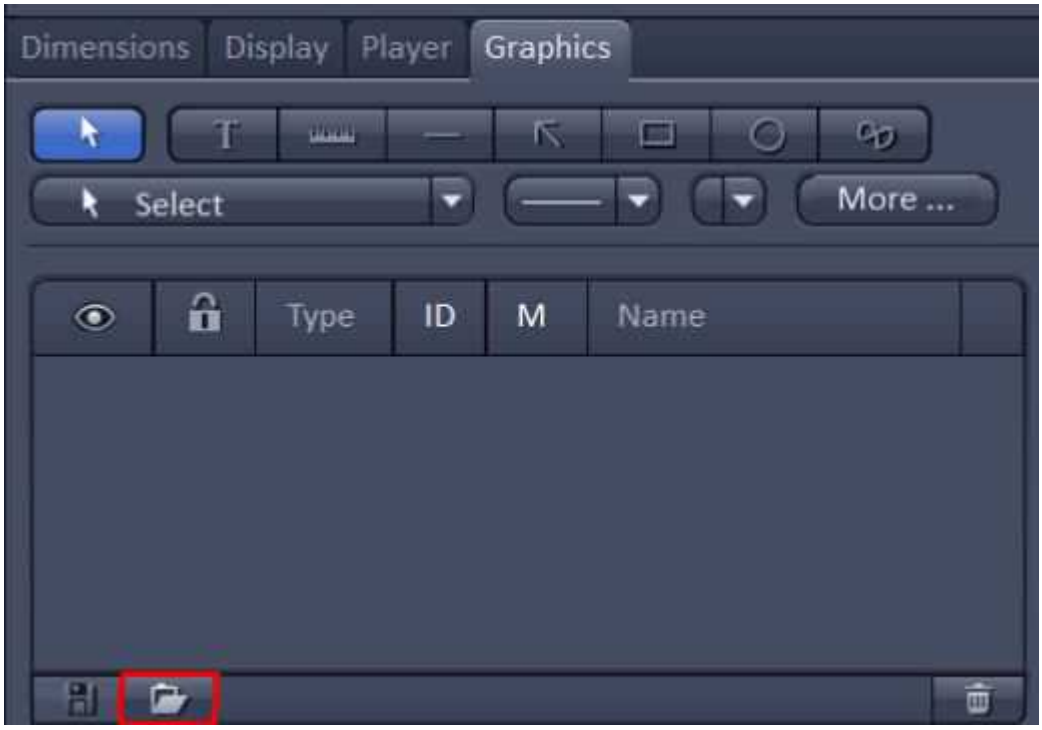
35.4 Go to Method -> Experimental PSF
- Select Filtered image via select button
- Adjust ROIs option
- Set appropriate parameters go to step #34 for advice



35.5 Go to Image Viewer -> Graphics -> Save button and save the ROIs

35.6 Go back to the PSF raw data image window
Go to Method -> Experimental PSF
- Select the raw data via the Select button
- Select manual ROIs

- Load saved selections via Image Viewer -> Graphics -> Load button

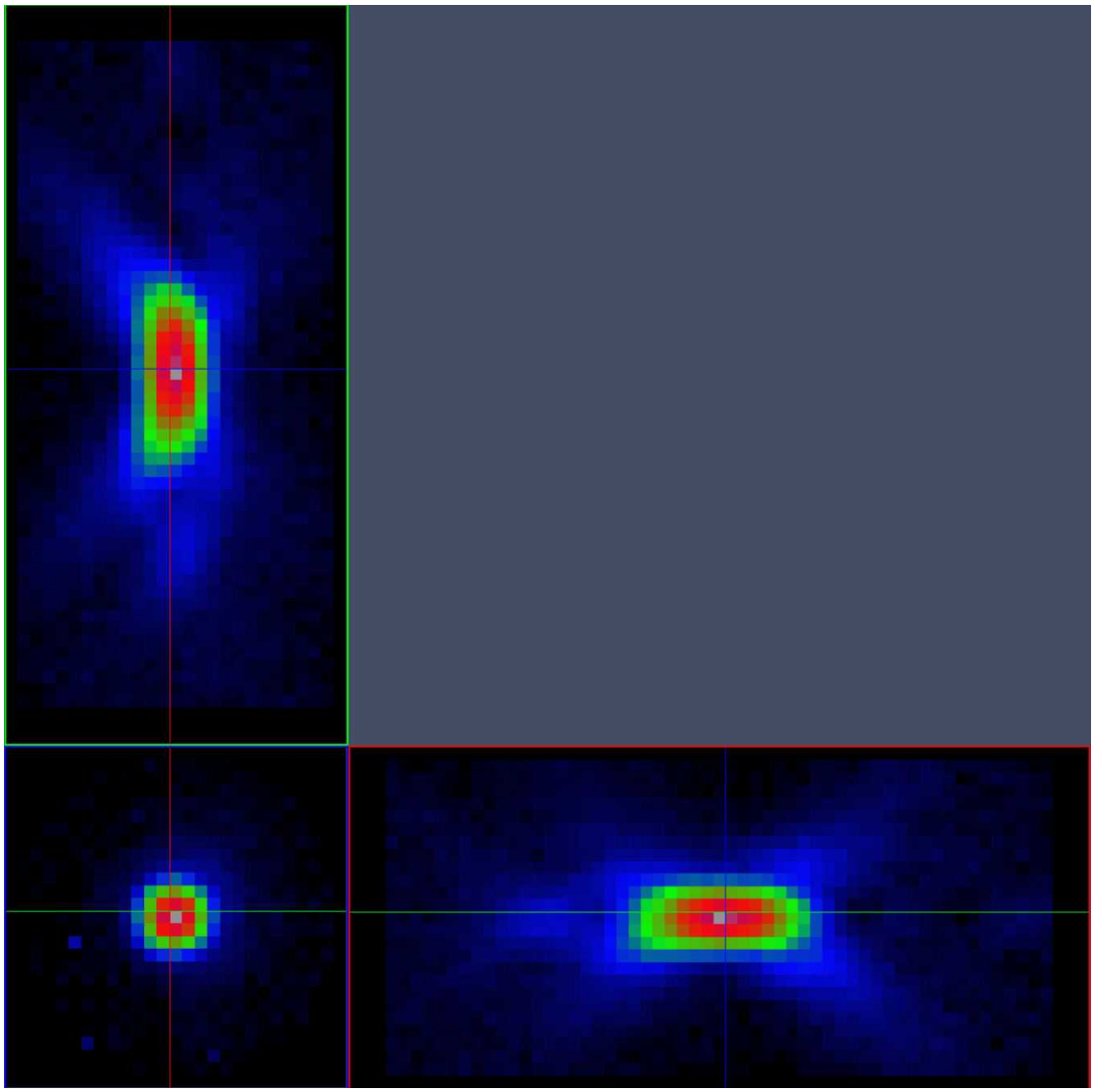


- Click on Apply

36 Inspecting PSF output:

- Orthogonal view with gamma correction can help you to find asymmetries in the PSF
- Quantitative results (PSF FWHM) can be found in the output's image info tab

Expected result



Orthogonal views

Notes	FWHM(X-Y) = 238 nm; FWHM(Z) = 560 nm; FWHM(X) = 238 nm; FWHM(Y) = 238 nm;

PSF FWHM information