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## Repeatability of the XY positioning of motorized xy-tables of light microscopes

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QUAREP-LiMi

Quarep-LiMi WG6



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### DISCLAIMER

The members of QUAREP-LiMi Working Group 6 developed this protocol. The member list can be found here: (<https://quarep.org/working-groups/wg-1-illumination-power/wg-6-members/>)

QUAREP-LiMi is a group of scientists interested in improving quality assessment (QA) and quality control (QC) in light microscopy. We first came together in April 2020; as of September 2023, the group has grown to 552 people from 39 countries worldwide. We have members from academia, microscopy communities, companies, organizations or institutions related to standardization, scientific publishers, and observers from funding agencies.

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

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## ABSTRACT

The time it takes for the entire microscope system to be in equilibrium with the environment is called the settling time and is highly affected by changes in environmental conditions. Long-term measurements only provide conclusive results of the system if the environmental conditions are stable over time. In particular, temperature fluctuations will influence drift measurements in all axes. Tracking the temperature of the microscope and the room is highly recommended for long-term measurements and to indicate the drift stability. This is particularly important for correctly interpreting the results and accurate measurements. Discussions among the working group experts have shown that allowing the microscope system to adapt and equilibrate to the room/measurement conditions for a specific time is common practice. The time for thermal equilibration with the room conditions can easily reach several hours. It is important to note that it is not always possible to wait such a long time before starting iterative measurement sessions, but at least the microscope system and room should be characterized initially and referred back to as the baseline environment measurement reference parameter. As with other QUAREP-LiMi protocols, a 1 hour equilibrium period should be observed, with minimal deviations in temperature and humidity.

## GUIDELINES

- The environmental conditions should be 22° and ideally constant temperature (air-conditioned).
- A short warm-up of the stage is good, e.g. a short random move over the whole travel range.
- If this is not possible, running the stage at least once over the whole travel range is still good. This has the advantage of evenly distributing all the lubricant on the spindle and guides. This also happens every time the stage is initialized.
- Regular maintenance by the stage manufacturer should be kept in mind. Depending on the running time, this can be different, as a guideline serves: With max. 8 hours per day at the latest after 5 years (different from stage manufacturer to stage manufacturer). A reference run can also be made occasionally to check the stage performance.

## MATERIALS

Use a sample of fluorescent beads such as 1µm, 4µm TetraSpeck, or FocalCheck beads attached to a coverslip, 35 mm petri dish or any other sample format used in the experiment.

- A microscope with a motorized x,y stage, a secure specimen (slide) holder, either an integrated motorized Z focus mechanism or a mechanized z-axis specimen holder.
- Standard glass microscope slide prepared with large fluorescent beads (e.g. TetraSpeck 1 or 4 µm diameter) mounted in appropriate mounting media and high precision #1.5 cover glass. See protocol of QUAREP-LiMi WG 5 for PFS slide preparation.
- Possibly, a glass slide with a precise etched pattern can also be used. e.g. “cell finder” (ibidi) or similar patterns. Other quality control test standard slides, such as the Argolight slide series, can also be used. However, the analysis workflow needs to be adapted.

## SAFETY WARNINGS




The stage will move several mm throughout the protocol in the x and y direction. Please ensure that mechanical interferences between the stage and sample with optical elements of the system (e.g., objective lenses) can be excluded during this movement.

## BEFORE START INSTRUCTIONS

1. Switch on the entire system well in advance before the measurement. Allow the system and stage to reach temperature equilibrium for at least 1 h before starting the measurement.
2. Ensure a thoroughly cleaned microscopy system.
3. Ensure the proper functioning of the entire microscopy system.
4. Ensure a stable room temperature. The maximal variation in temperature needs to be smaller than 1.0 degrees over the measurement period. If possible, track the temperature next to the stage. Maintain room temperature vs. stage surface temperature data for each calibration session using one data logging room temperature and humidity sensor and another data logging temperature probe located on the stage very near the specimen slide or vessel holder.
5. Ensure the system is protected from environmental disturbances (e.g. vibrations, heavy airflow, etc.). Eliminate sources of temperature variation and any vibration sources that affect sub-micron positioning.
6. If not doable, a run overnight is an alternative.
7. Ensure that the sample is rigidly mounted in the insert and that the insert is properly fixed to the stage to prevent movements caused by the shift of the sample. Give the sample enough time to be in thermal equilibrium with the room temperature.
8. Ensure that the microscope room door is not opened/closed many times during the acquisition to minimize any vibrational or temperature-related fluctuations. If needed, put a note on the door saying, 'Do not enter. Imaging in progress.'

## Acquisition

- 1 When starting the acquisition software, first initialize (if applicable) the motorized stage platform in the XY direction.
- 2 Please make sure that the objective turret is positioned (lowered) so that none of the objectives can make contact with the stage platform and be damaged by this initialization procedure. This is an absolute caution step for commercial microscopes and particularly for custom build set-ups.
- 3 Select a 10x or 20x dry lens and select the XYT acquisition mode of the system control software. Any drift control of the instrument itself must be disabled.

#### Note

The precision of the measurement will be determined by the numerical aperture of the objective lens and the lateral sampling frequency (image pixel size in  $\mu\text{m}$ ). To check the reproducibility of the stage the use of dry objectives is recommended and the resolution they are providing is sufficient for  $r$  measurements of the stage. However, the protocol can also be used with immersion objectives if the measurements shall be used to ensure experiment reproducibility. For measurements with any immersion media fixation of the sample is key to obtaining meaningful results.

#### Note

The speed acceleration values of the stage shall resemble the default values of the stage when used in a typical experiment. Any change in these values must be documented.

- 4 Test that the stage insert as well as the sample is securely fixed. Any sample/insert movement will deteriorate the measurement.

- 5 Position a fluorescent bead in the center of the image. It is acceptable that other beads are within the field of view as well. If necessary, adjust the acquisition parameters. Adjust the exposure time of the detector to avoid saturated pixels within the image as this will impact the subsequent analysis. For camera-based systems, the maximal pixel intensity of the bead equals roughly 50% of the camera dynamic range, i.e. around 128 grey values for an 8-bit acquisition and 2048 for a 12-bit image. For point-scanning confocal systems, it is recommended to use the entire dynamic range.

It is recommended to use Nyquist sampling for point-scanning confocal systems and no binning for camera-based detection systems.

#### Note

For the measurement, it is essential that the bead is well attached to the surface and is not moving during the experiment. Multiple beads in the field of view and/or repeating the measurement can serve as a control to ensure the beads are well attached to the surface.

## Acquisition

**6** Record the stage positions. The set of positions is called one cycle.

**Note**

Some acquisition software allows setting/changing the travel speed of the stage. Routine repeatability measurement shall be performed at the default travel speed of the stage. If the protocol is used with varying stage speeds, this needs to be documented, as the stage speed might affect the repeatability.

**6.1** Record/store the position with one bead in the center of the FOV (P1, if possible, mark as "0") in the acquisition software.

**6.2** Shift the stage so the bead moves 300  $\mu\text{m}$  to the left (-300  $\mu\text{m}$  in x). Record the position (P2).

**Note**

A deviation of several  $\mu\text{m}$  is acceptable when setting the stage positions.

**6.3** Return to the initial bead position. Record the position again (P3).

**Note**

Some microscope acquisition software will not allow you to mark the same exact position twice. As we will only measure the shift in P1 mark as close to P1 as the software allows.

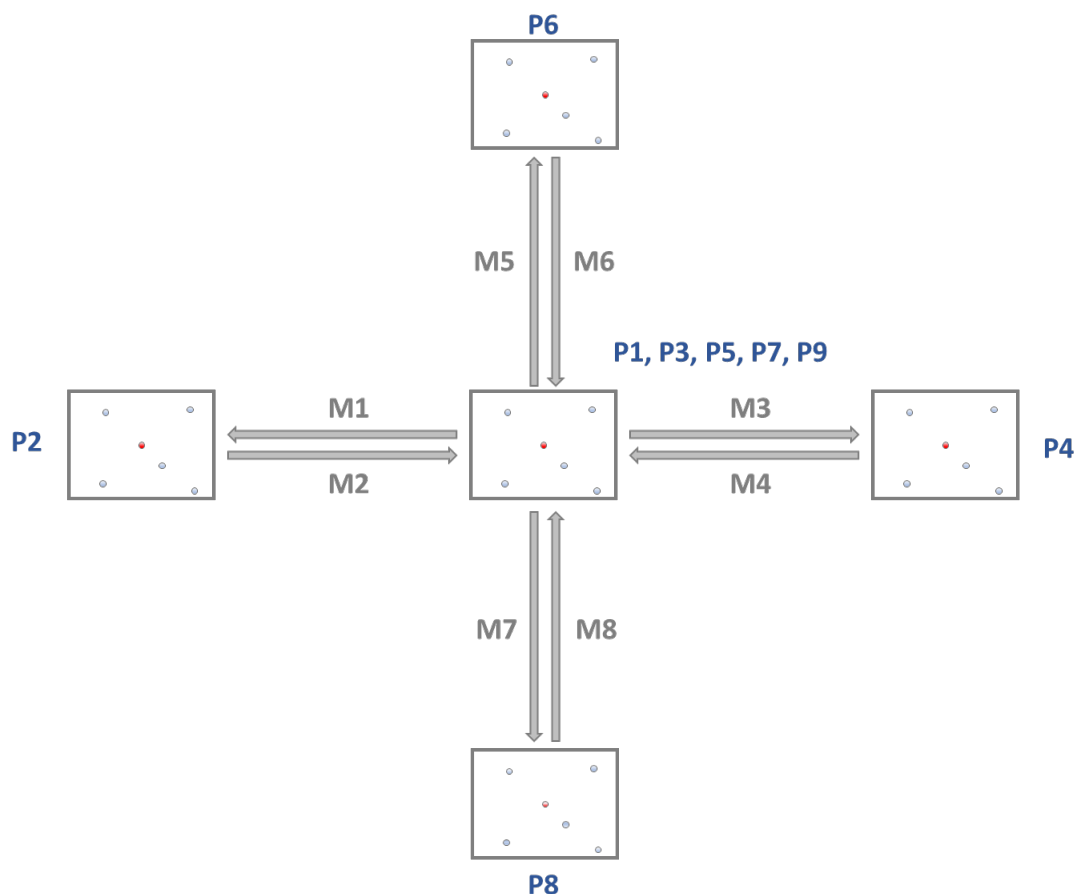
**6.4** Shift the stage so the bead moves 300  $\mu\text{m}$  to the right (+300  $\mu\text{m}$  in x). Record the position (P4).

**6.5** Return to the initial position. Record the position again (P5).

- 6.6** Shift the stage so the bead moves 300  $\mu\text{m}$  to the top (+300  $\mu\text{m}$  in y). Record the Position (P6).
- 6.7** Return to initial (center) position. Record the Position again (P7).
- 6.8** Shift the stage so the bead moves 300  $\mu\text{m}$  to the bottom (-300  $\mu\text{m}$  in y). Record the position (P8).

**Note**

Some microscope acquisition software will not allow you to mark the same position twice. In this case, it is also possible to omit the positions P3, P5, P7, and P9.



Schematic representation of the measurement scheme. The recorded positions are called one cycle of repeatability measurements. Only the central bead (red) will be

used for the analysis. Other beads in the field of view can be tolerated as long as they have a distance of  $> 10\ \mu\text{m}$  from the central bead.

- 7 Set up a multi-position time-lapse experiment (XYT) with 20 cycles (one cycle corresponding to the nine previously defined positions). Thus the stage travels to each position 20 times (=20 measurement cycles) and acquires an image at each position. The cycles shall be scheduled without any further delay.

#### Note

For the subsequent analysis, only the images of position P1 will be taken into account. Image acquisition at the other points can be omitted if the acquisition software allows doing so.

- 8 Repeat the whole procedure for a displacement distance of  $4000\ \mu\text{m}$  (4mm).

#### Note

The current protocol tests the stage's repeatability issues on two scales ( $300\ \mu\text{m}$  and  $4000\ \mu\text{m}$ ). These scales had been established with stage manufacturers and shall be sufficient to report on typical issues with stages. However, nothing speaks against adding a third scale (e.g.  $50\ 000\ \mu\text{m}$ ) if the experiment requires it. In that case, care must be taken that the stage can travel that distance without hitting other objectives.

## Analysis

- 9 The following analyses are based on Fiji.  
Screenshots are made from ImageJ 2.14.0/1.54f; Java 1.8.0\_172 [64-bit].  
Step 9 includes a Step case.  
**Quantitative analysis**  
**Qualitative analysis**  
**Validate sample preparation**

step case

### Quantitative analysis



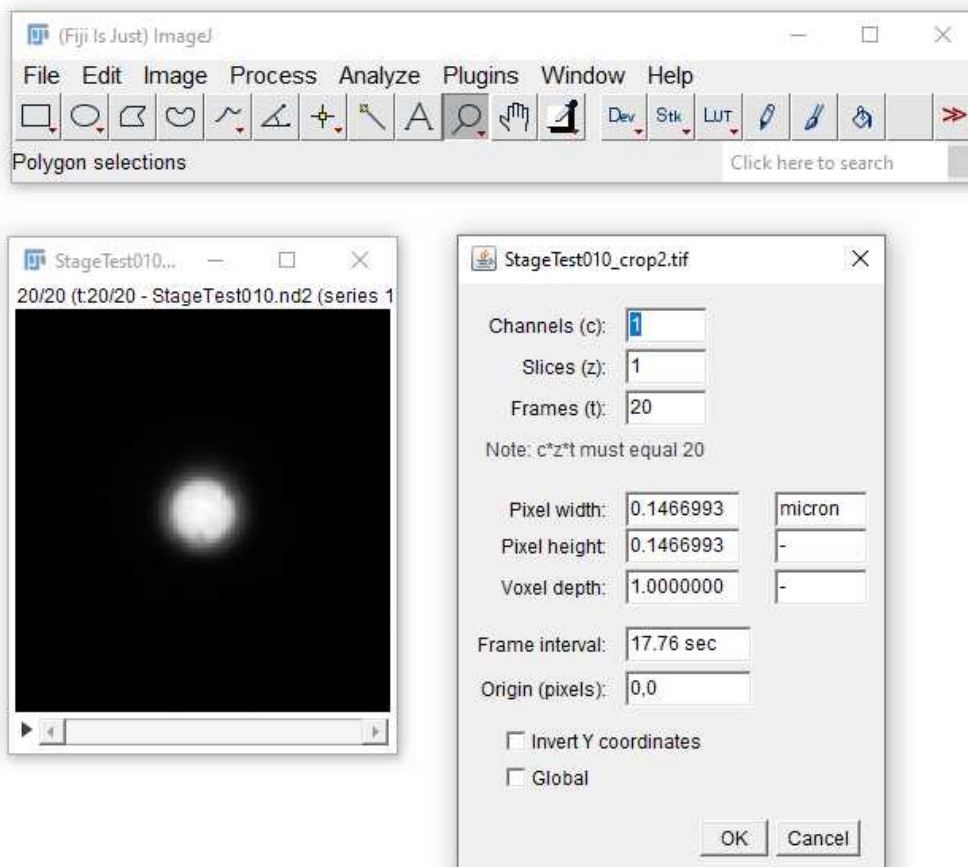
## 10 Open image in Fiji.

### 10.1 Open image sequence/time lapse of Position 1 in Fiji/ImageJ.

#### Note

For the subsequent analysis, only the images of position 1 will be considered. The exact workflow of how to achieve this will differ slightly from one acquisition software to the next. However, no major hurdles are expected as long as the file format is supported by OME-bioformats.

### 10.2 Check that the image is properly calibrated, i.e., the pixel width and height correspond to the acquisition settings. This information is typically automatically stored in the image's metadata for commercially available systems. The calibration can be checked in the menu Image-->Properties... in Fiji (see image below)

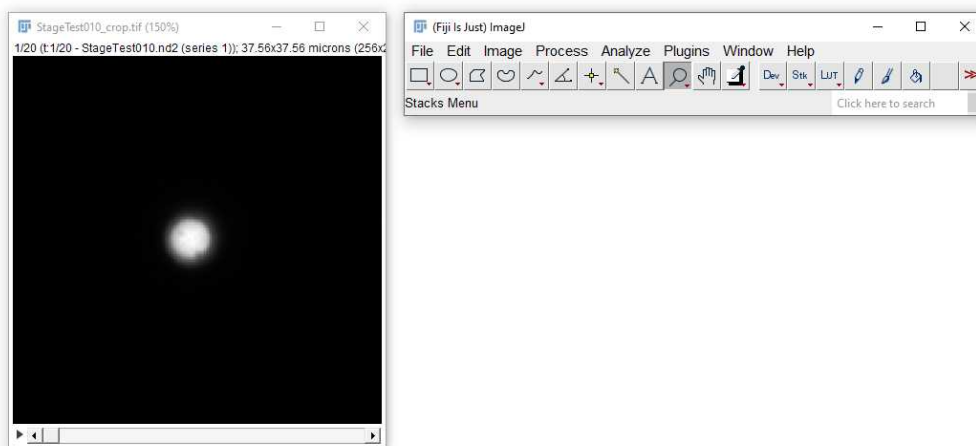


### 10.3 Crop the image so the resulting window only contains a single bead, preferentially the most

centred one.

### Note

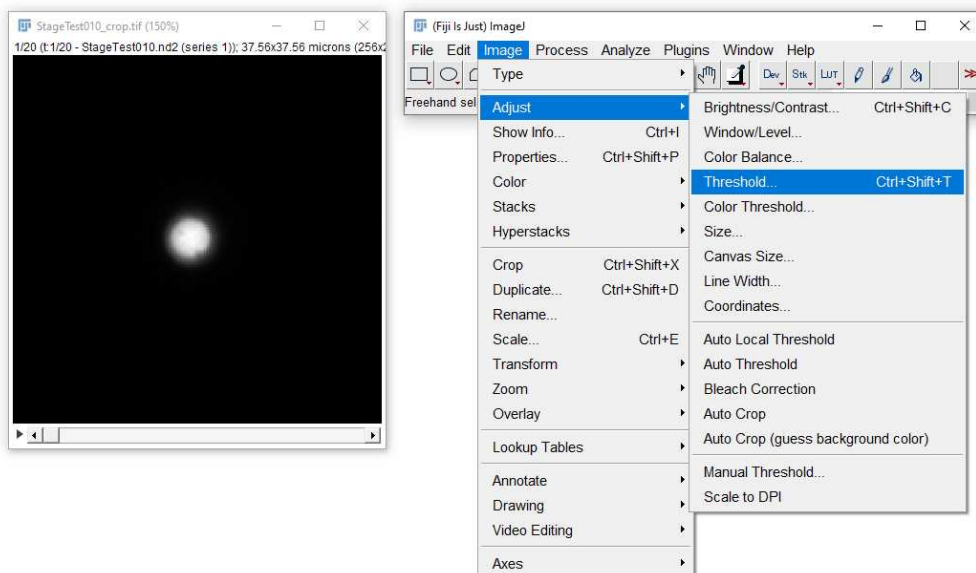
The size of the cropped region will not influence the analysis as long as there is only one bead per field of view.



Example of an image stack (20 frames) cropped to a region with exactly one bead per field of view displayed with Fiji.

## 11 Image Segmentation

### 11.1



Adjust the threshold of the image to detect/segment the bead using the method "Li" as detailed in the next step.

## 11.2

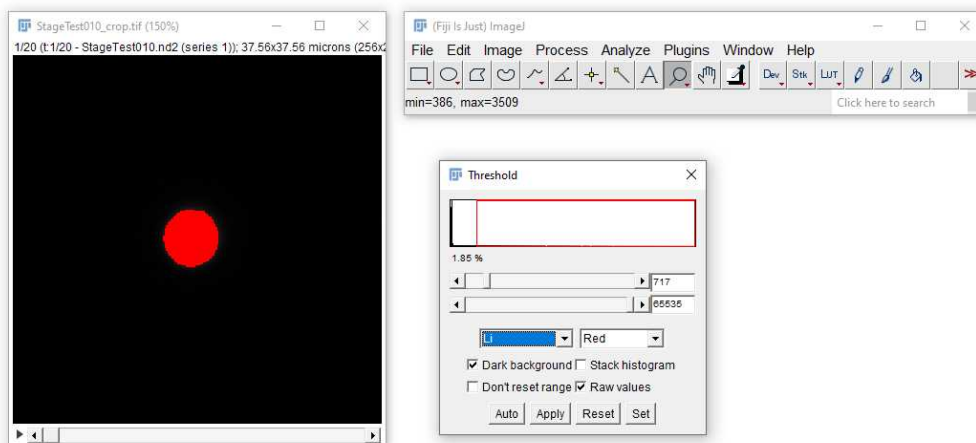


Image -> Adjust -> Auto Threshold: Method "Li", Check "Dark background"  
 Check that the bead is properly detected by the selected thresholding method.  
 It is essential that per field of view, only one particle is detected.

## Note

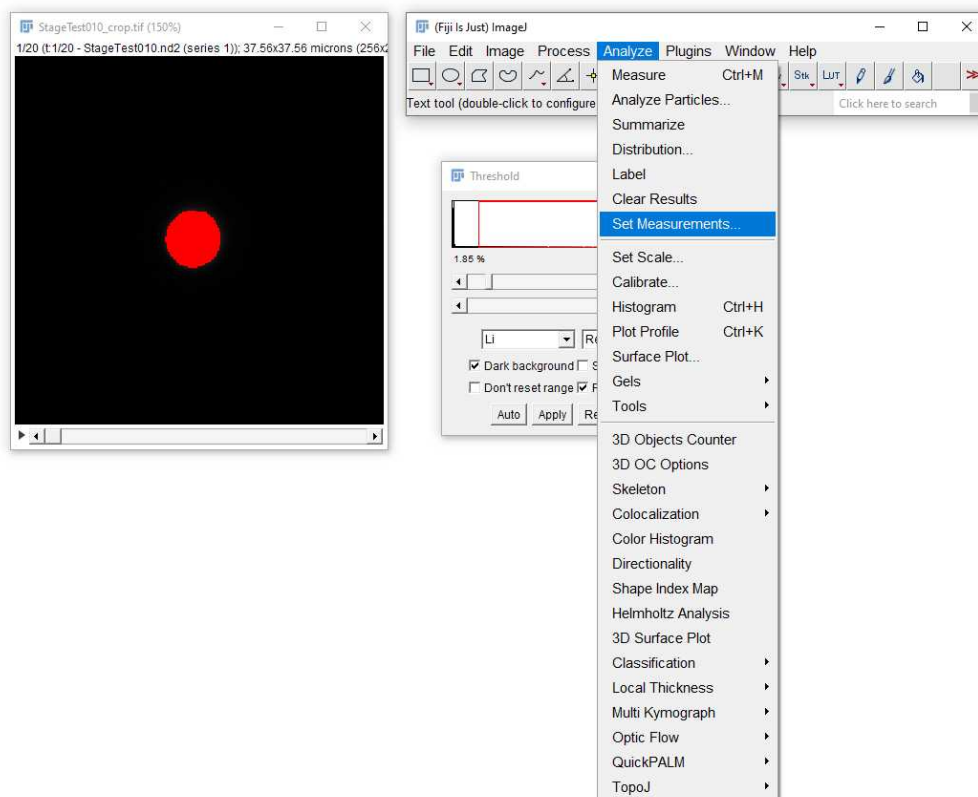
Adjusting the threshold manually is not recommended. If the described method is not working, it might be worth checking/modifying the settings for the image acquisition.

## Note

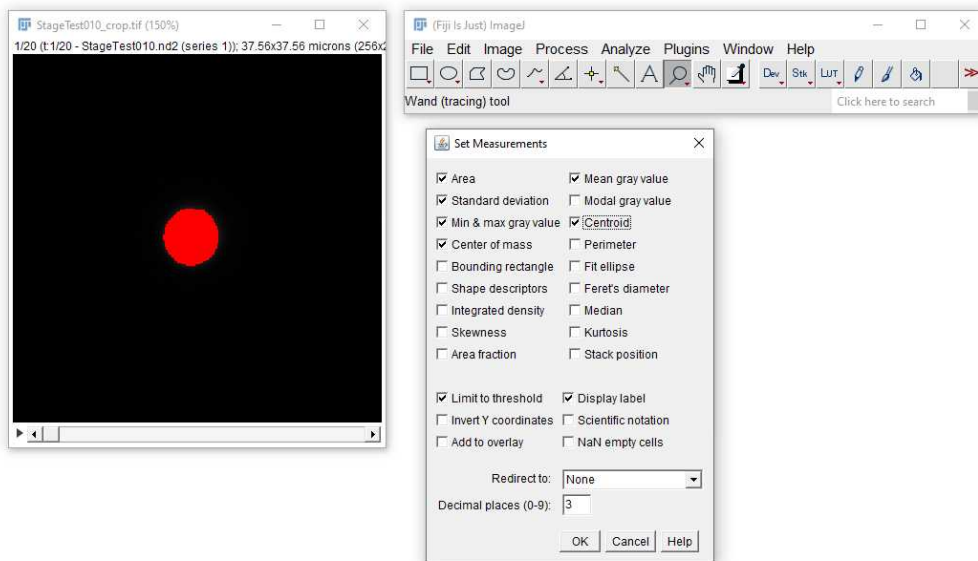
Only one connected area must be detected. If the thresholding routine results in more than one area, the image can be filtered using the Mean (Process-->Filters-->Mean...) or the Gaussian blur (Process-->Filters-->Gaussian Blur...) filter.

## 12 Analyse the position of the bead.

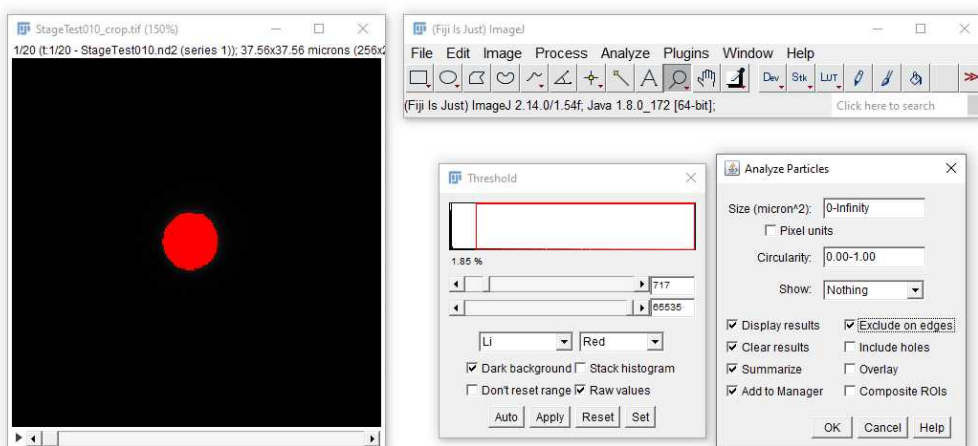
### 12.1



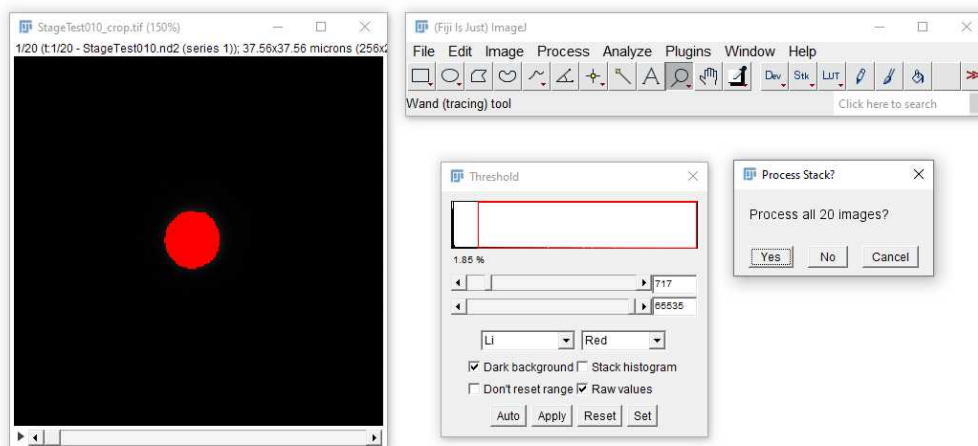
"Set Measurements..." is used to specify the parameters to measure (see the following screenshot).



## 12.2

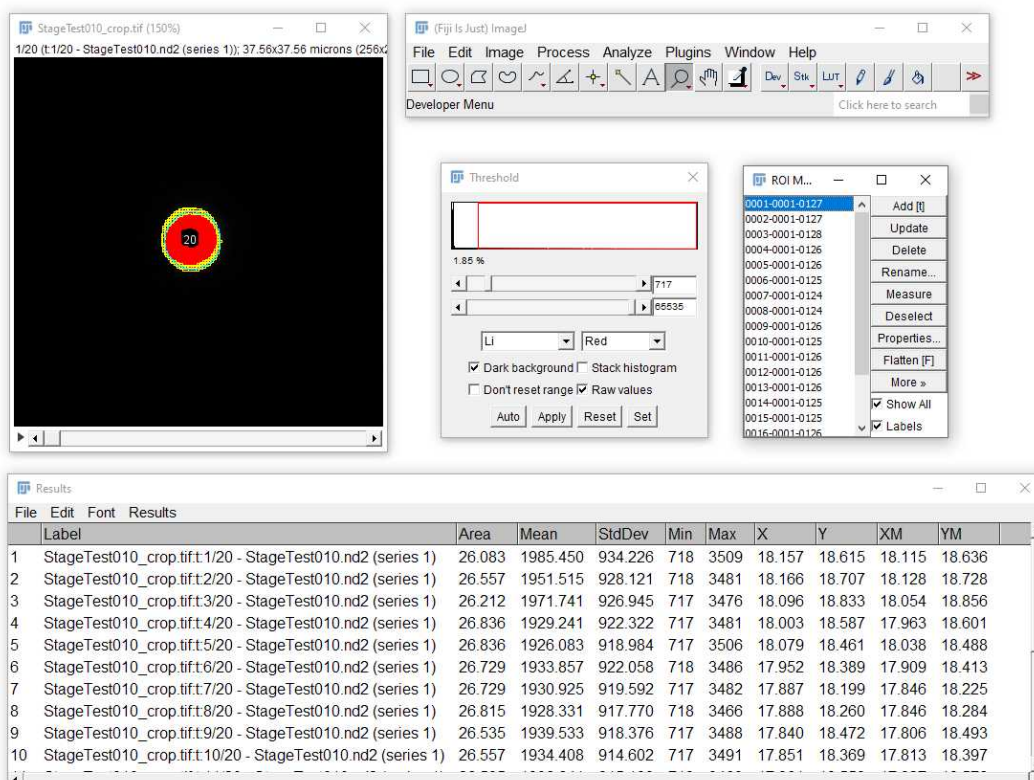


Analyze -> Analyze Particles



You need to confirm that the entire stack will be analysed.

## 12.3



The obtained table from the "Analyze Particles" operation. To report on the stage performance, columns X, Y, XM and YM are the most relevant. The table can be saved as

a ".CSV" file.

- 13** To report on the performance of the stage, calculate the standard deviation of all X and Y positions. These values can be used to report on the stage performance in the field over time.