

# Multifocal Image Analysis – User Guide

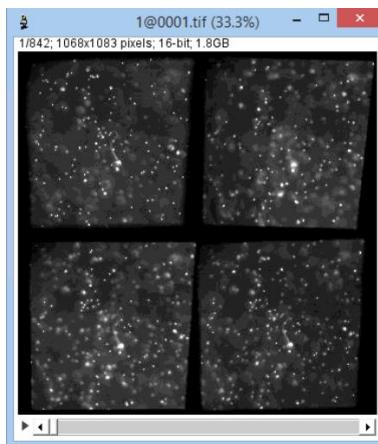
## Introduction

This user guide describes how to use and apply software published along the manuscript **Multifocal imaging for precise, label-free tracking of fast biological processes in 3D** (Jan N. Hansen, An Gong, Dagmar Wachten, René Pascal, Alex Turpin, Jan F. Jikeli, U. Benjamin Kaupp, Luis Alvarez. bioRxiv 2020.05.16.099390; doi: <https://doi.org/10.1101/2020.05.16.099390>). For more information, detailed license notes, an installation guide, etc. visit <https://github.com/hansenjn/MultifocalImaging-AnalysisToolbox>.

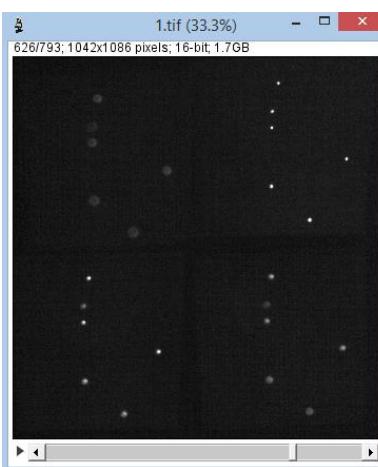
## 3D Bead tracking

### Requirements

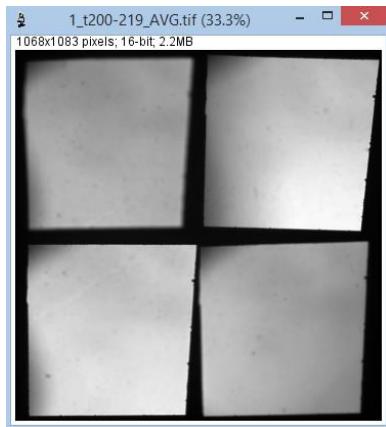
1. Multifocal imaging recording of beads



2. A multifocal imaging piezo-generated z stack through exemplary beads to obtain a calibration between bead width and z position



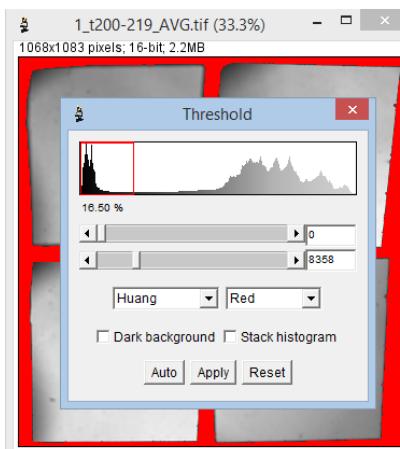
3. A reference file for intensity corrections (obtained by imaging without a specimen)



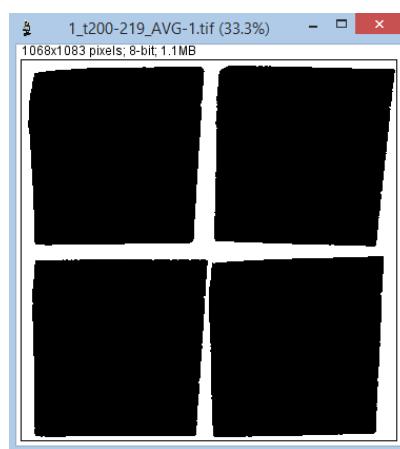
## Workflow

### Creating an intensity heat-map

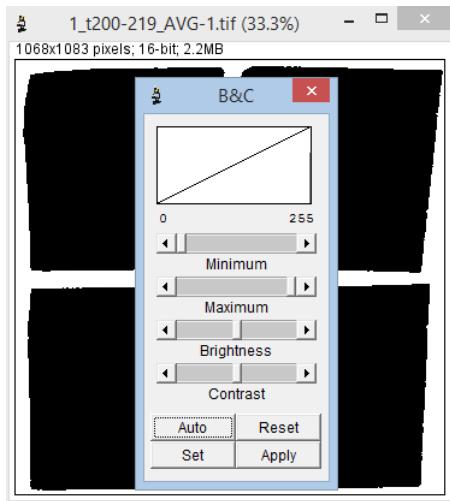
- Open the reference file for intensity corrections in ImageJ
- Duplicate the image: Image > Duplicate
- Binarize the duplicate: Image > Adjust > Threshold, select Huang, press Apply



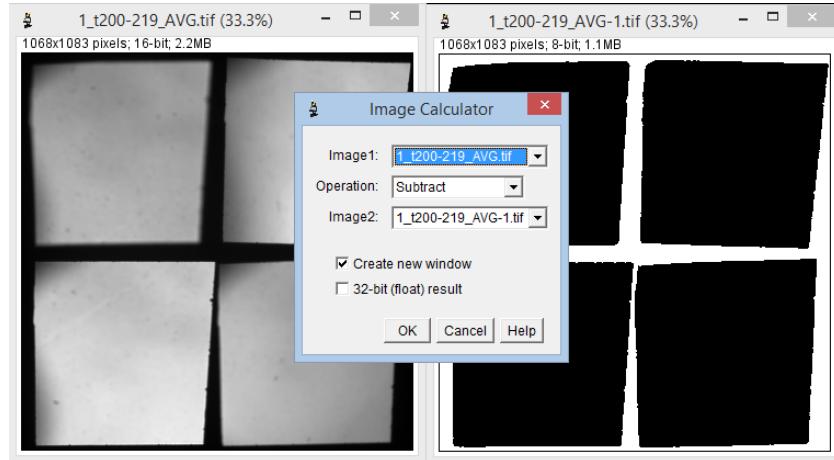
- The duplicate image will look like this:



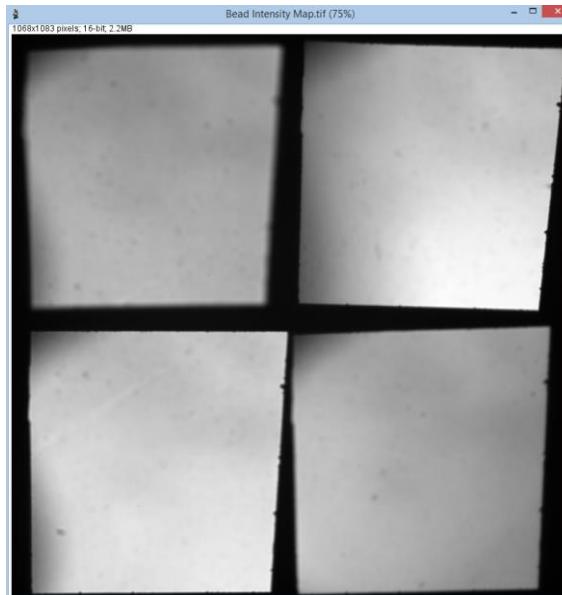
- Convert the image to 16-bit: Image > Type > 16-bit
- Reset the intensity values: Image > Adjust > Brightness & Contrast, Press Apply



- Remove the non-plane areas from the map: Process > Image Calculator, Select the operation “Subtract”, the raw image as Image1 and the binarized duplicate image as Image2, Press OK

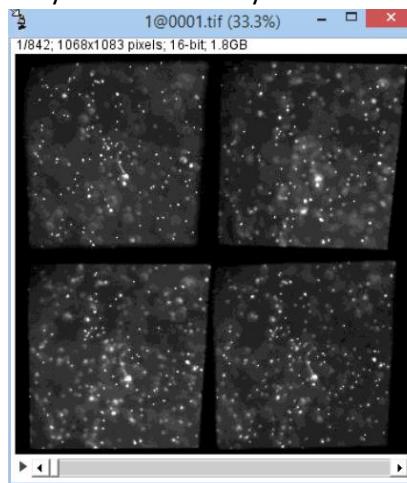


- Save the generated image as “Bead Intensity Map.tif”, it will serve as a map for intensity corrections in the plugin “Multifocal Preparation”.

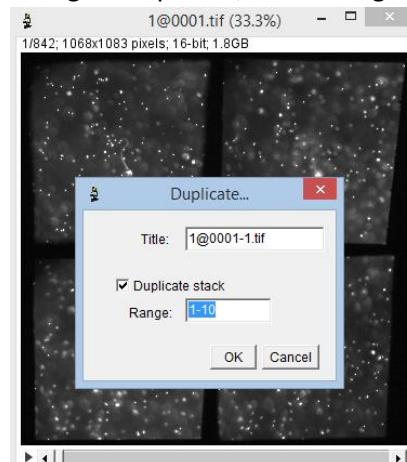


### Creating a registration file

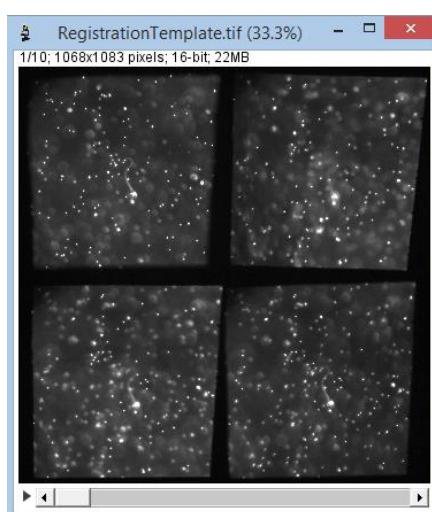
- Install the plugin MultiStackReg from Brad Busse: <http://bradbusse.net/downloads.html>
- Install the latest release from the plugin Multifocal\_Preparation: [https://github.com/hansenjn/MultiFocal\\_Preparation/releases](https://github.com/hansenjn/MultiFocal_Preparation/releases)
- Restart ImageJ
- Open the multifocal time series you want to analyze



- Duplicate the first 10 frames: Image > Duplicate, select Range 1-10, press OK

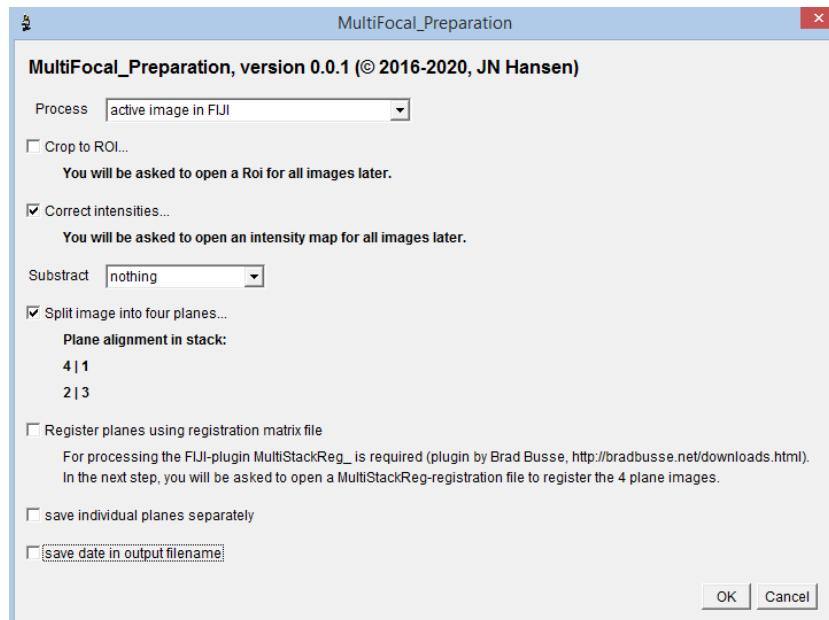


- Save the image as "RegistrationTemplate.tif"

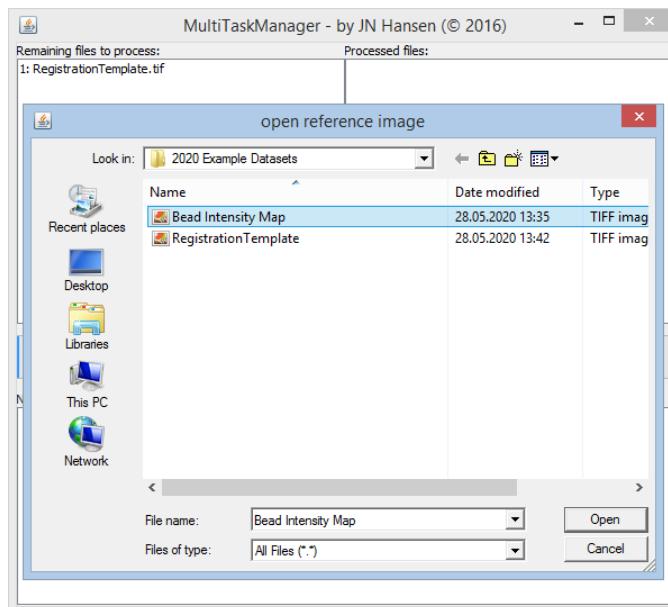


More information, license notes, credits: <https://github.com/hansenjn/MultifocalImaging-AnalysisToolbox>

- Process the image with MultiFocal\_Preparation: Plugins > JNH > Multi Focal > Prepare raw data for analysis, select the following options and press OK.



- In the upcoming dialog, select the “Bead Intensity Map.tif” produced before as a reference image:

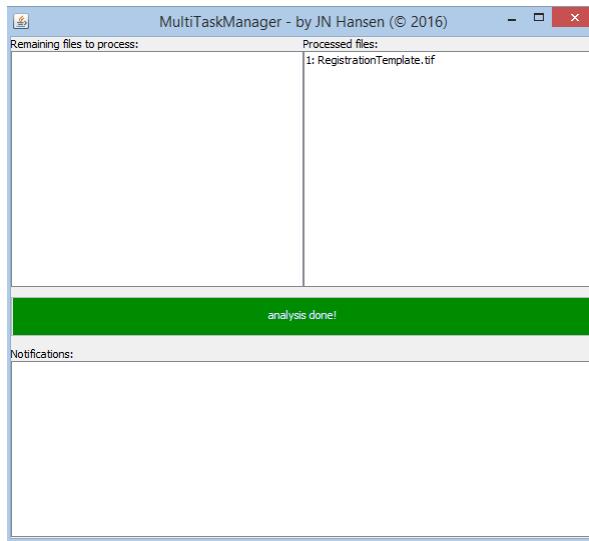


- Wait until the Processing is Done
  - In brief, the plugin will correct the intensities according to the reference image and then split the image into 4 equal quarters
  - Each quarter becomes a separate plane in the output Hyperstack
  - The conversion is as follows: the upper right quarter becomes plane 1, the lower left quarter plane 2, etc. as indicated in the settings dialog:

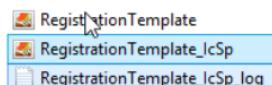
Plane alignment in stack:  
4|1  
2|3

- When the Processing is Done, the MultiTaskManager dialog looks like this:

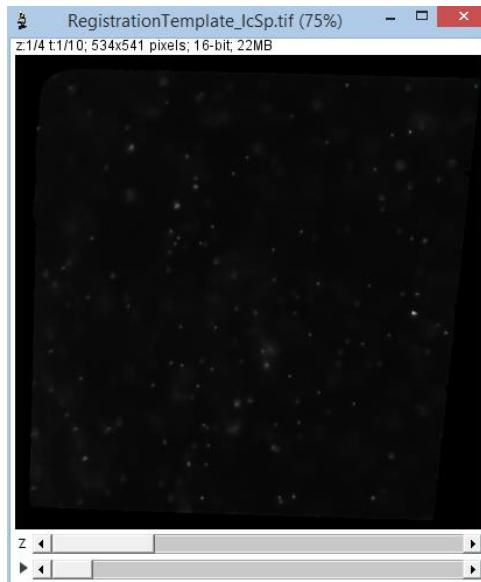
More information, license notes, credits: <https://github.com/hansenjn/MultifocalImaging-AnalysisToolbox>



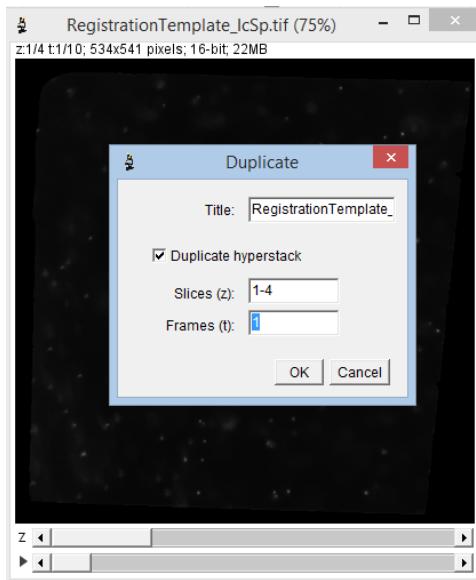
- Then, new files have been saved to the repository where the RegistrationTemplate.tif file was saved.



- The .tif-file with ending IcSp contains the output Hyperstack, the text file documents the processing settings of “MultiFocal\_Preparation”.
- Open the RegistrationTemplate\_IcSp.tif file in ImageJ



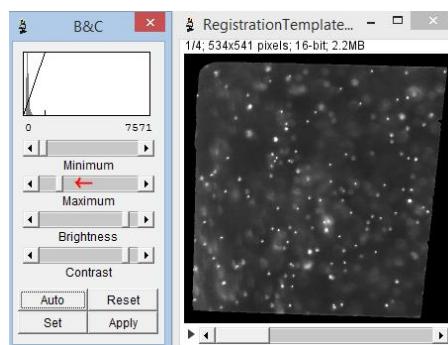
- You can see that each focal plane has become a different Z slice.
- Extract a single timepoint: Image > Duplicate, select the following settings, press OK



- A new image pops up containing only one time point and the four different planes

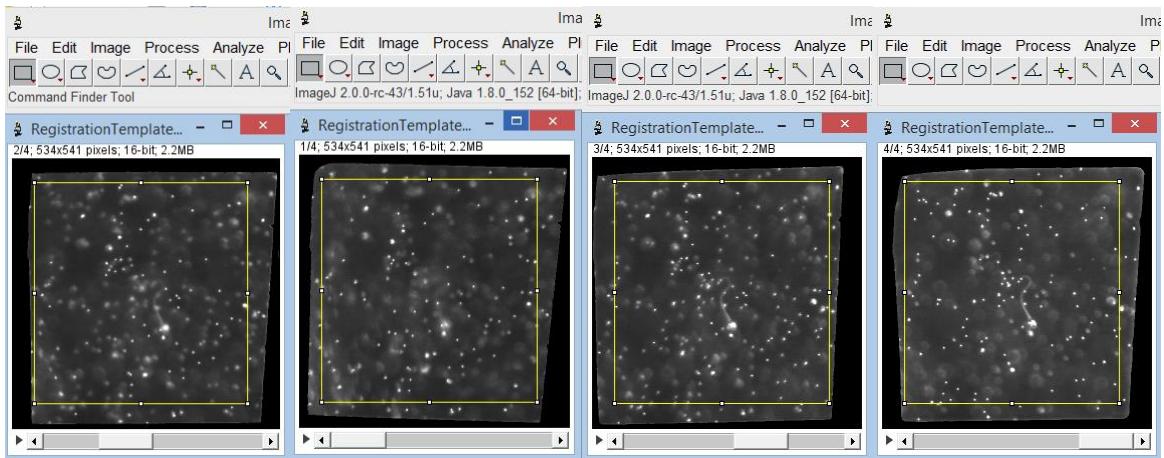


- Adapt the display range so that you can see the borders of the planes: Image > Adjust > Brightness/Contrast, drag down the maximum so that edges become apparent in the image

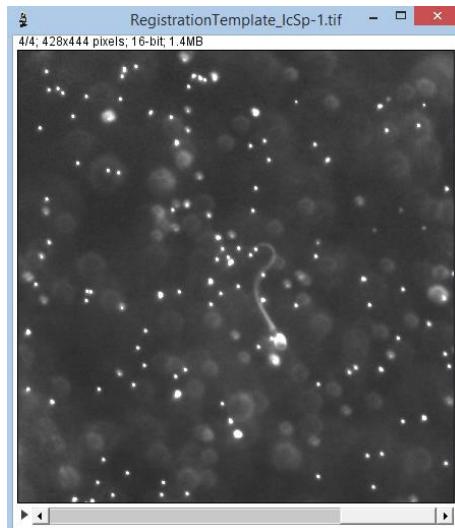


- Select the Rectangle tool in the ImageJ bar and draw a selection that is inside all planes and does not contain any black areas. Check the different planes by scrolling with the mouse wheel and adapting the selection.

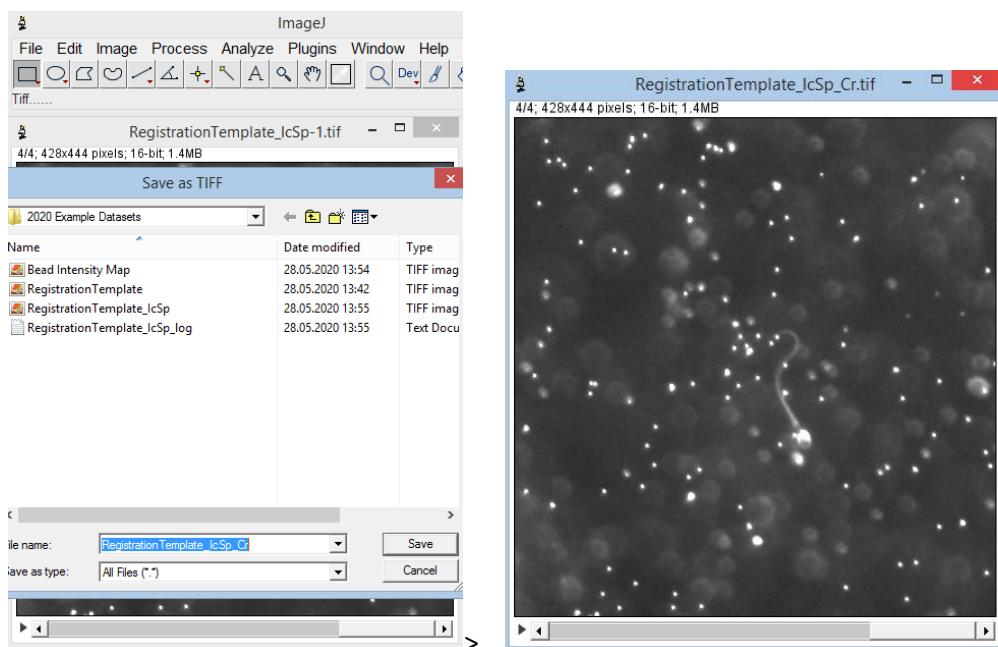
More information, license notes, credits: <https://github.com/hansenjn/MultifocalImaging-AnalysisToolbox>



- Crop the stack to the selection: Image > Crop

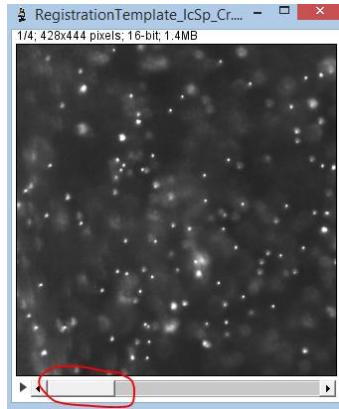


- Save the stack as RegistrationTemplate\_IcSp\_Cr: File > Save As ... > Tiff

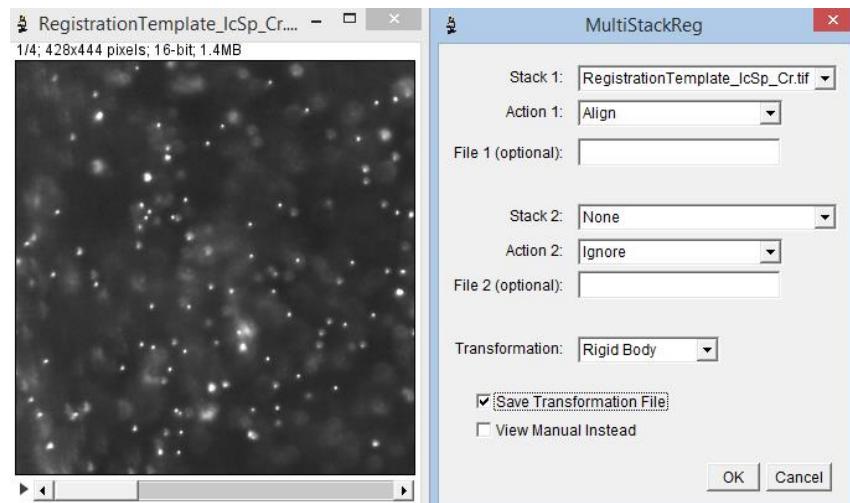


More information, license notes, credits: <https://github.com/hansenjn/MultifocalImaging-AnalysisToolbox>

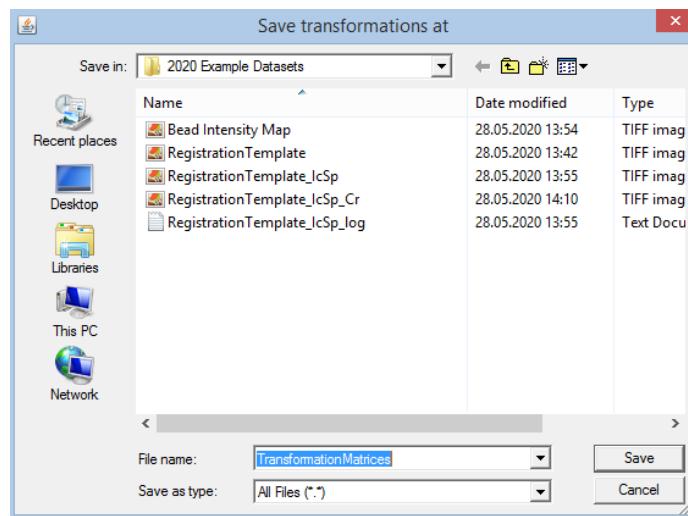
- Make sure that the first plane is selected



- Register the file: Plugins > Registration > MultiStackReg, select the following settings, press OK



- A dialog pops up, save the transformation matrices to the folder where the image was located

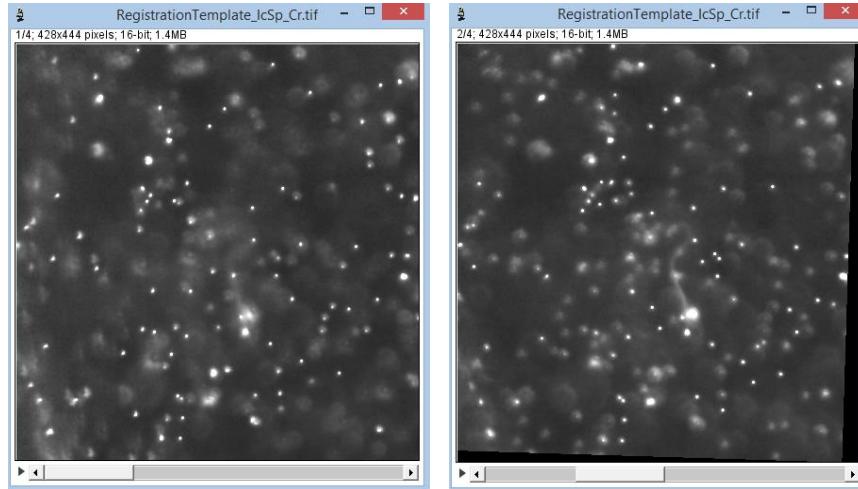


- Wait until the plugin is done and the status bar looks “resting”/normal again

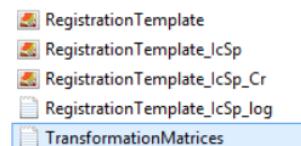


More information, license notes, credits: <https://github.com/hansenjn/MultifocalImaging-AnalysisToolbox>

- The planes in the image now have been aligned and the transformation matrix file has been saved
  - Scroll through the image to check whether the alignment is good
  - Compare for example plane 1 and plane 2:

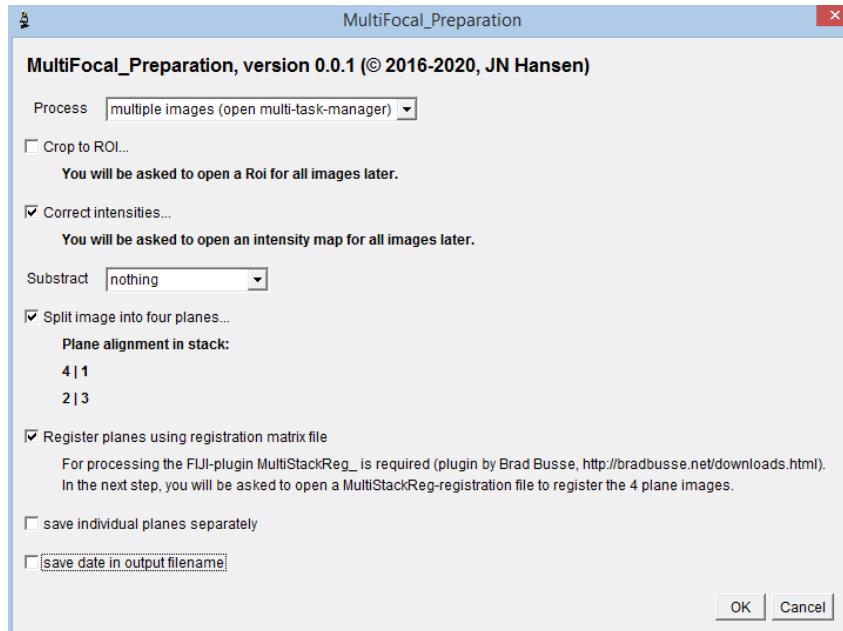


- The matrix file will be needed as a reference for registering the planes using the MultiFocal\_Preparation plugin.

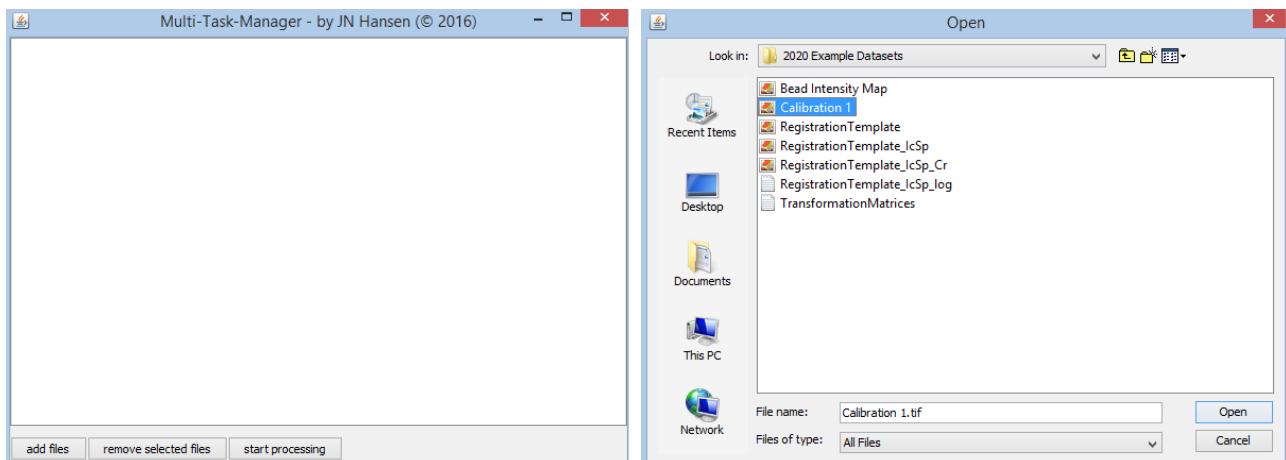


## Preparing the data for calibration and analysis (intensity correction, splitting, registering)

- Process all data that you want to use for calibration or that you want subject to analysis as follows with Multifocal\_Preparation: Plugins > JNH > Multi Focal > Prepare raw data for analysis; select the following options and press OK.

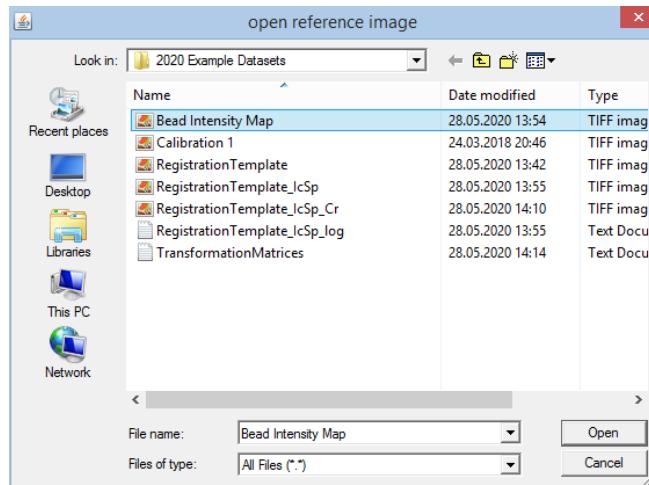


- A dialog pops up, press add files, select the files you want to analyze in your file system, press open (eventually repeat to add more files from different repositories), press start processing to let MultiFocal\_Preparation prepare the data.

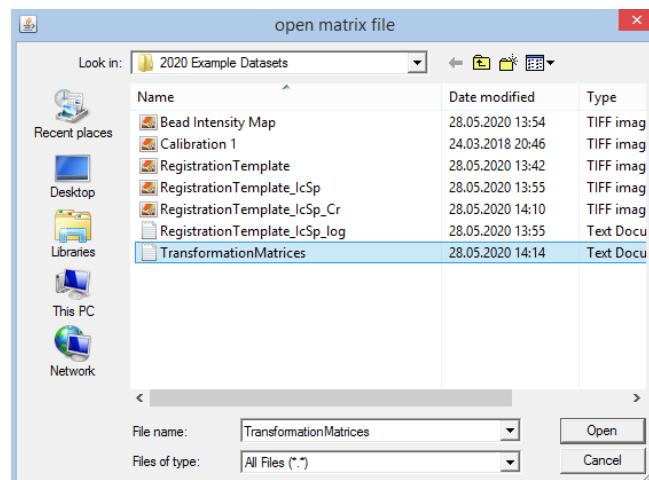


- A dialog pops up, select the Bead Intensity Map as a reference image and press open:

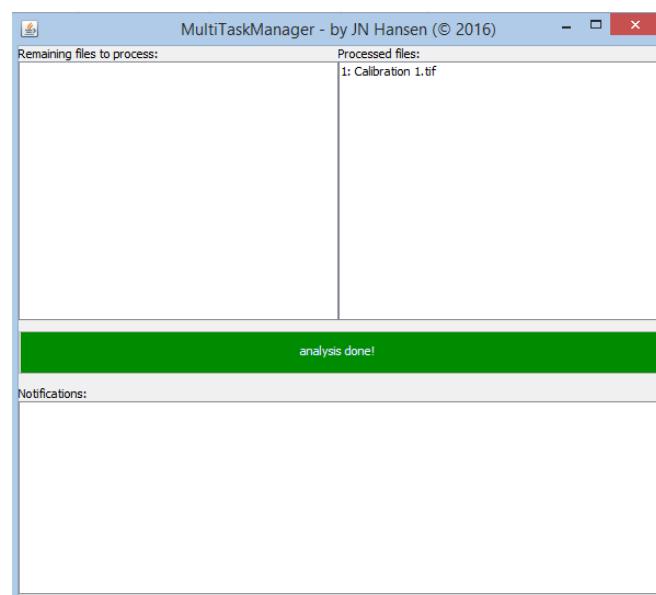
More information, license notes, credits: <https://github.com/hansenjn/MultifocalImaging-AnalysisToolbox>



- Another dialog pops up: Select the TransformationMatrices file and press Open.

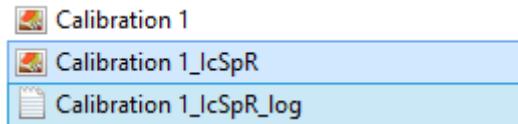


- To avoid any processing errors, don't touch the computer while processing. Some images might pop up and be hidden again during registration. Wait until processing is done.



More information, license notes, credits: <https://github.com/hansenjn/MultifocalImaging-AnalysisToolbox>

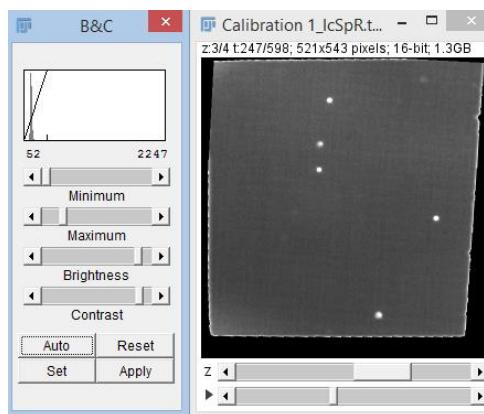
- At the location where the template files were saved, the output files will be saved. They receive an additional ending (\_IcSpR).



- The .tif-file with ending IcSpR contains the output Hyperstack and serves as a template for data analysis, the text file documents the processing settings of “MultiFocal\_Preparation”.

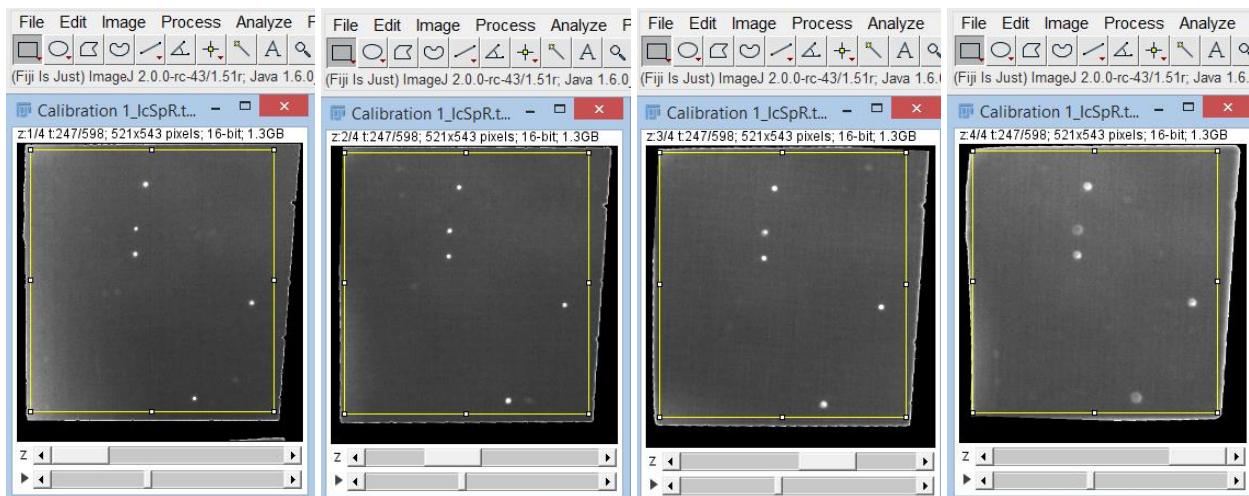


- Before analysis of the IcSpR image, crop the image to regions that are depicted in all four plane images:
  - Adapt the display range so that you can see the borders of the planes: Image > Adjust > Brightness/Contrast, drag down the maximum so that edges become apparent in the image

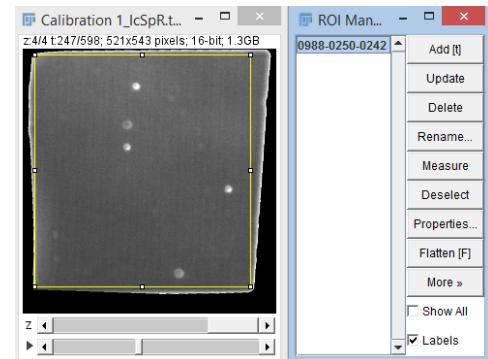


- Select the Rectangle tool in the ImageJ bar and draw a selection that is inside all planes and excludes all black areas. Check the different planes by scrolling with the mouse wheel and adapting the selection.

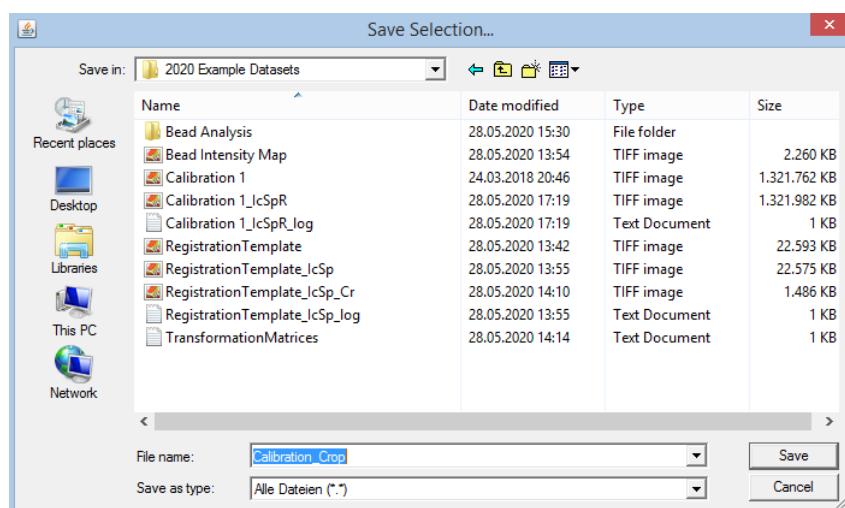
More information, license notes, credits: <https://github.com/hansenjn/MultifocalImaging-AnalysisToolbox>



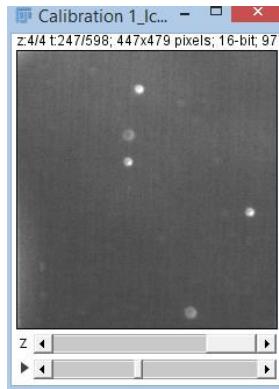
- You may save the selection for later use / reproduction of the image processing
  - Transfer the selection to the ROI Manager by pressing “t”



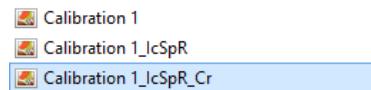
- Select the ROI in the ROI Manager and save it via More >> Save.



- Click on the image Window and crop the stack to the selection: Image > Crop



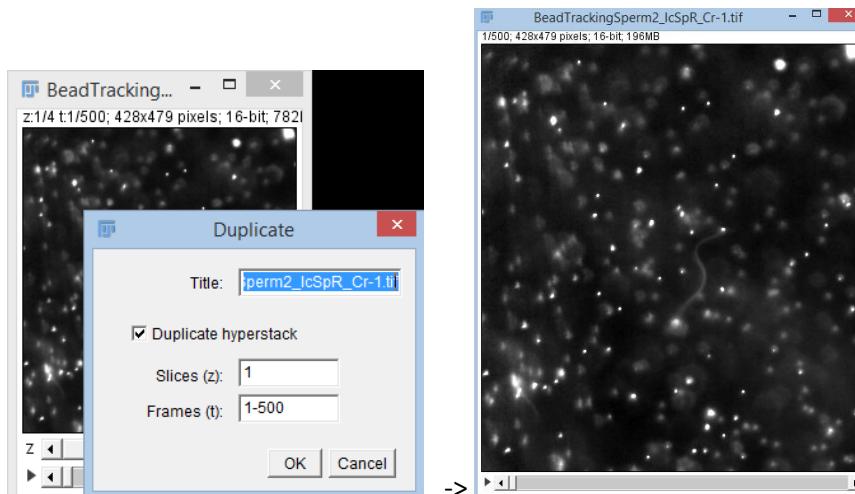
- Save the stack with the ending “\_cr.tif” to your directory: File > Save As > Tiff...



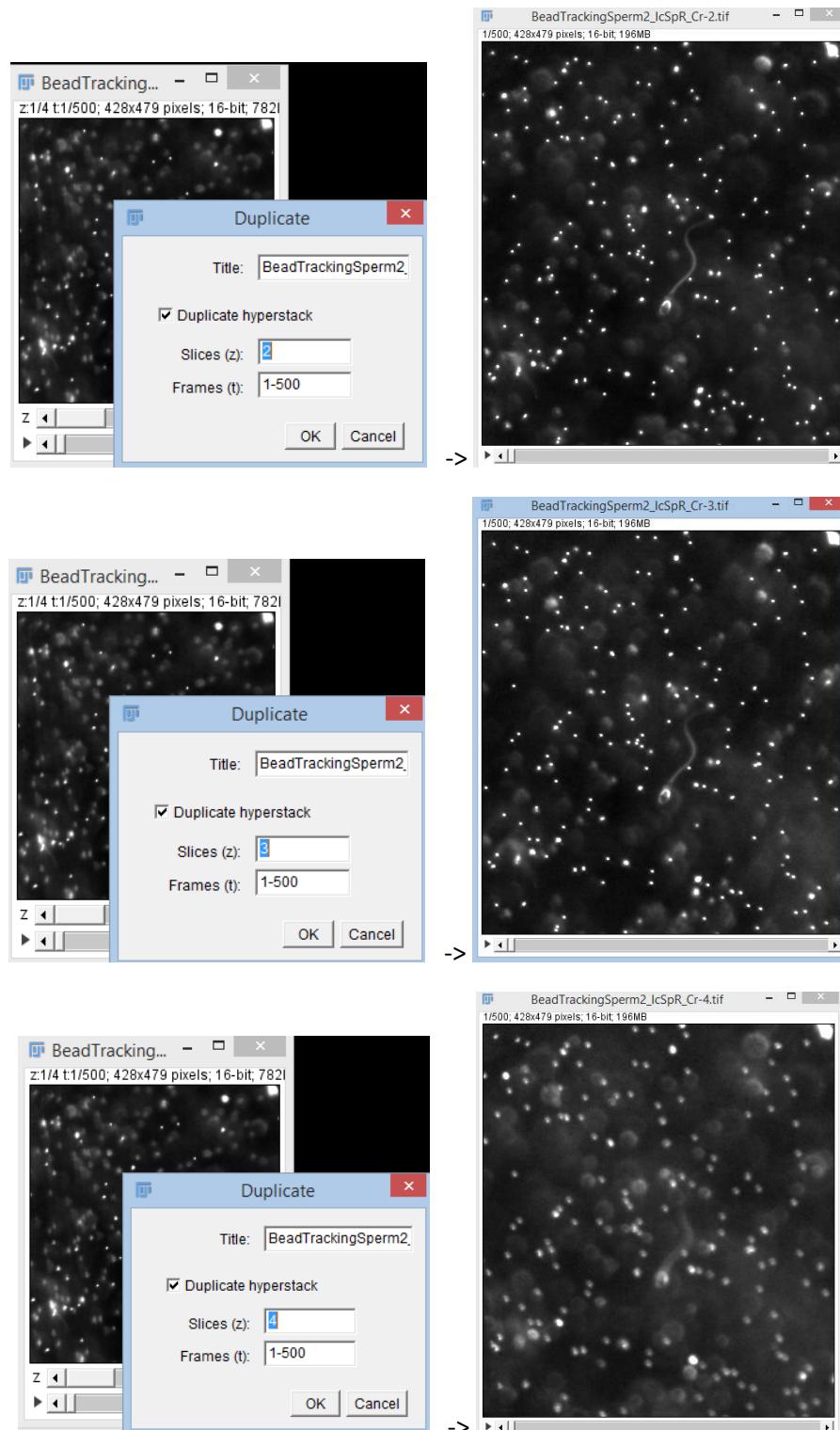
#### *Background reduction for images recorded for bead tracking or sperm tracking*

For images not used for the calibration process but for time-lapse bead tracking or sperm tracking, it might eventually help to subtract the background of the image via subtracting a time-average of the image sequence from the image sequence. Thereby, non-moving particles and background particles are removed from the image and do not disturb tracking. To do this, perform the following:

- If not still open, open the image saved as above (ending “...LcSpR\_Cr.tif”). Create image sequences for each individual plane by duplication: Click on the opened image, Image > Duplicate, select slice 1, press OK, click again on the opened Image, Image > Duplicate, select slice 2, press OK, ... (repeat the same with slices 3 and 4).

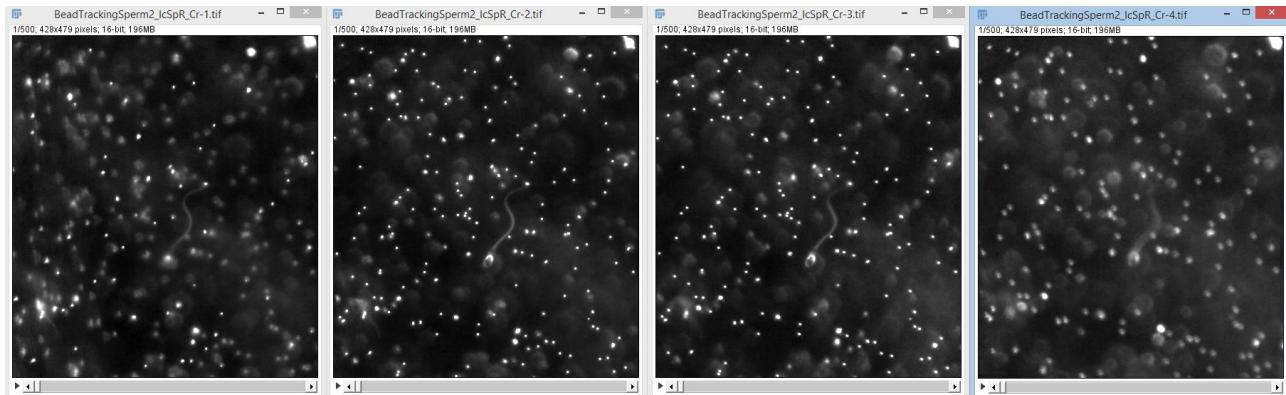


More information, license notes, credits: <https://github.com/hansenjn/MultifocalImaging-AnalysisToolbox>

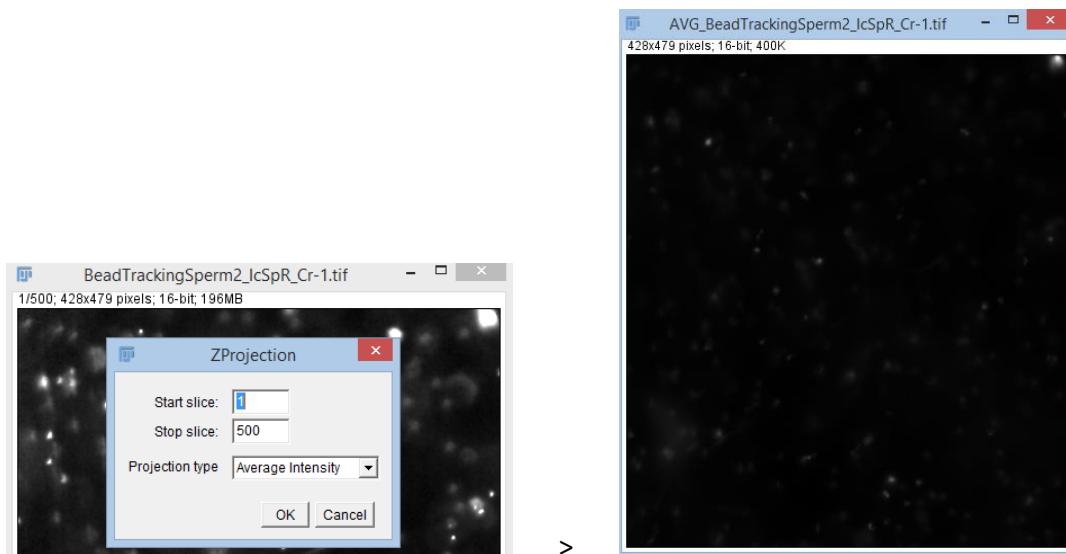


- Afterwards you should have an image for each of the four planes open in ImageJ:

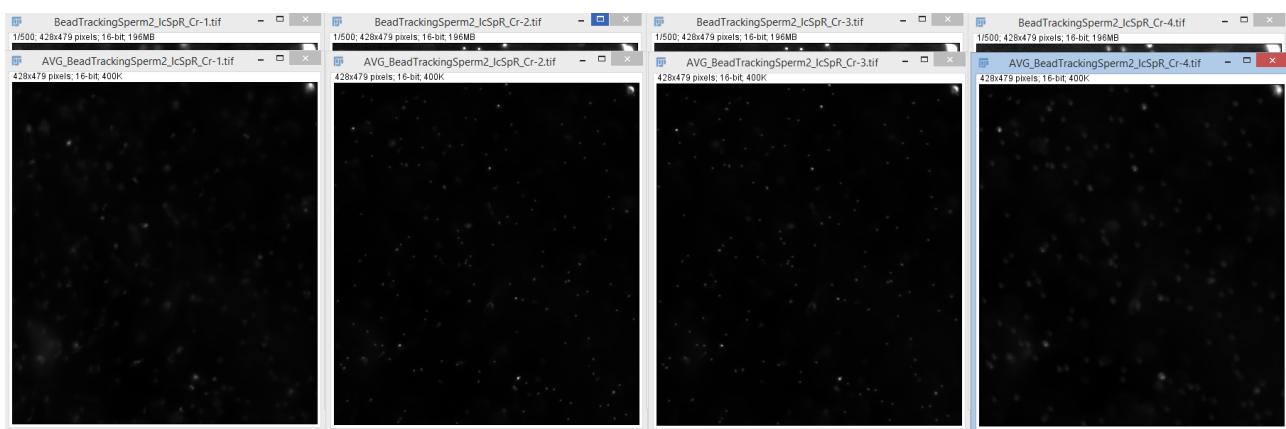
More information, license notes, credits: <https://github.com/hansenjn/MultifocalImaging-AnalysisToolbox>



- For each of those images, create an average projection: Click on the plane image, Image > Stacks > Z Project..., select “Average Intensity” as a “Projection Type”, press OK.
  - E.g. for the image of plane 1:

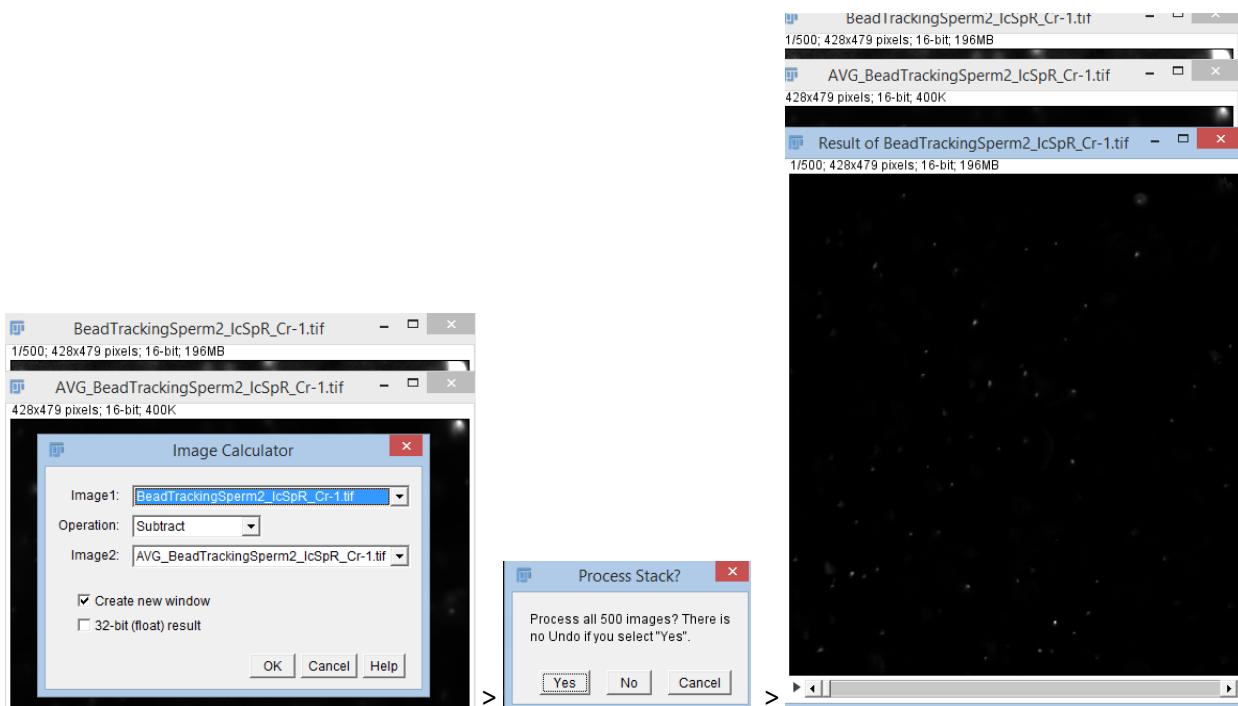


- Afterwards you should have an AVG projection for each plane image'

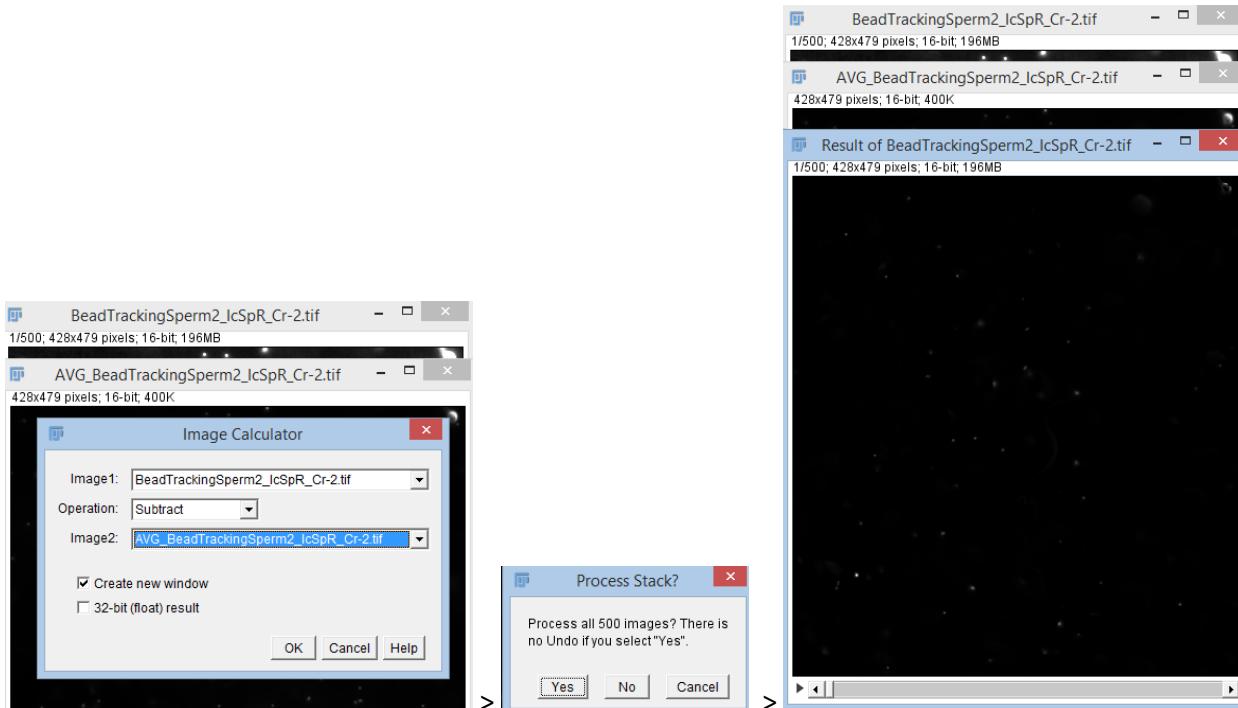


- For each plane image, subtract the respective AVG\_... projection image from the image series: Process > Image Calculator, select the image series as Image 1 and the respective AVG projection image as Image 2, press OK, press Yes in the upcoming “Process Stack?” dialog.
  - Plane 1:

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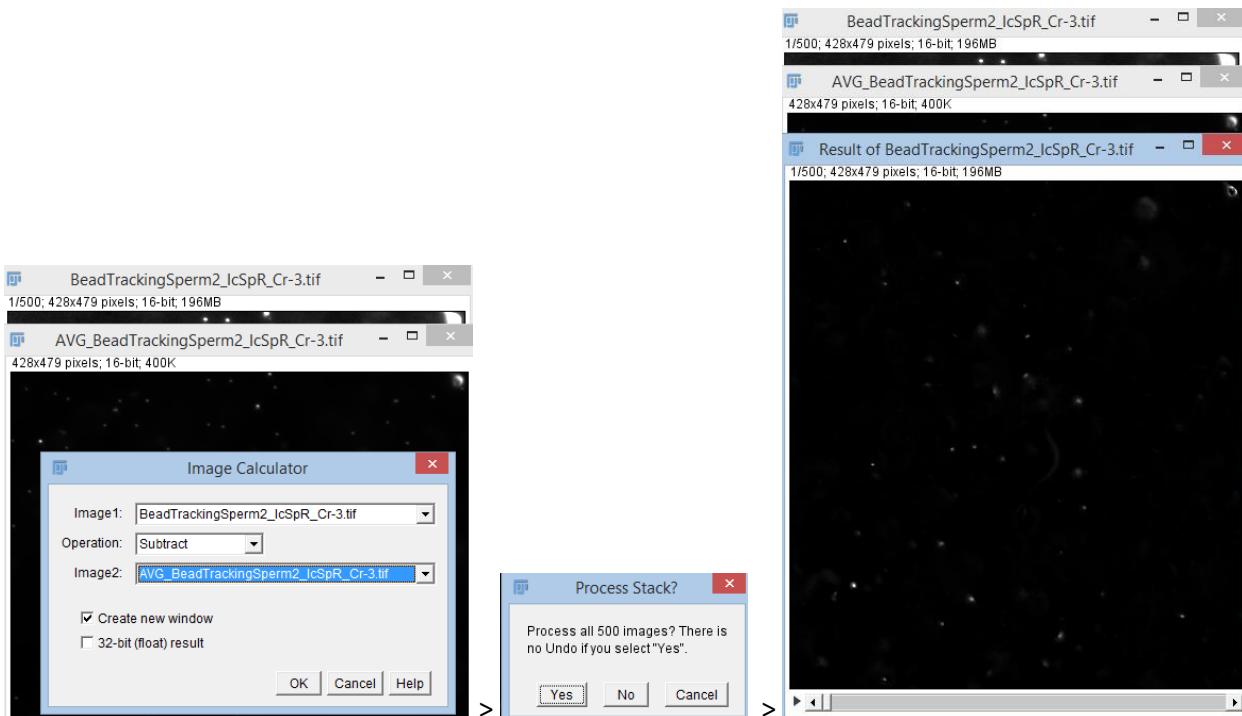


○ Plane 2

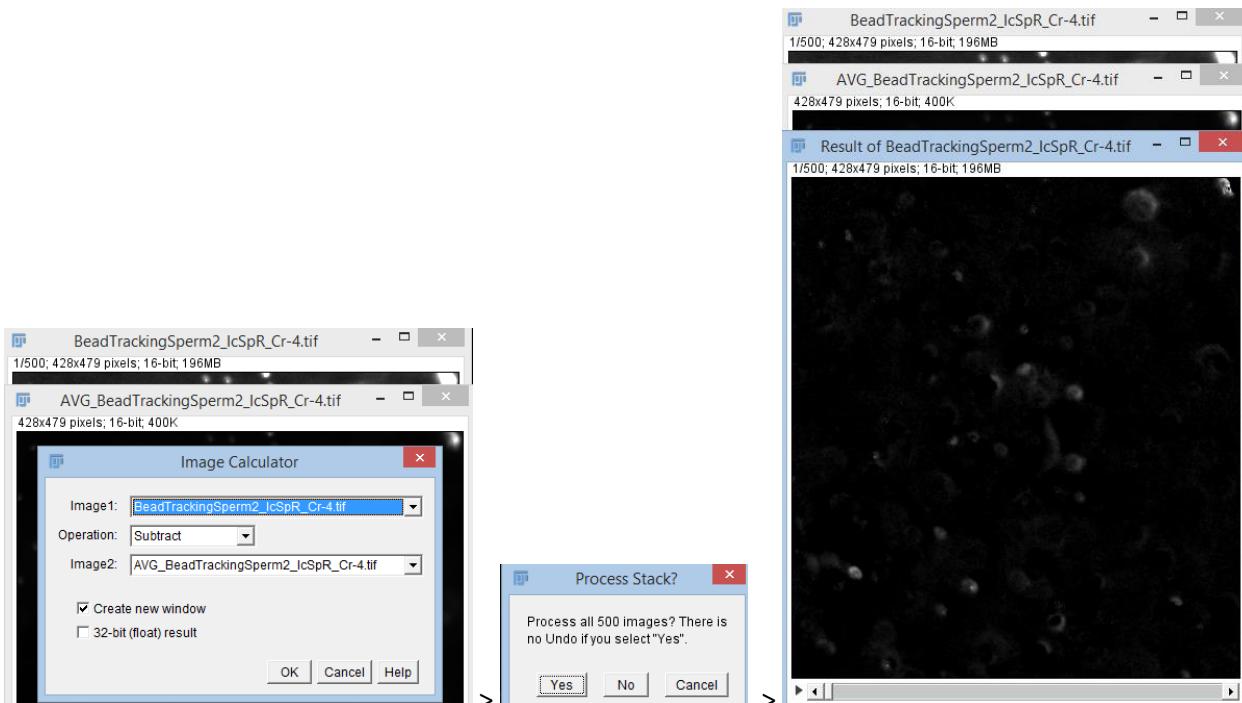


○ Plane 3

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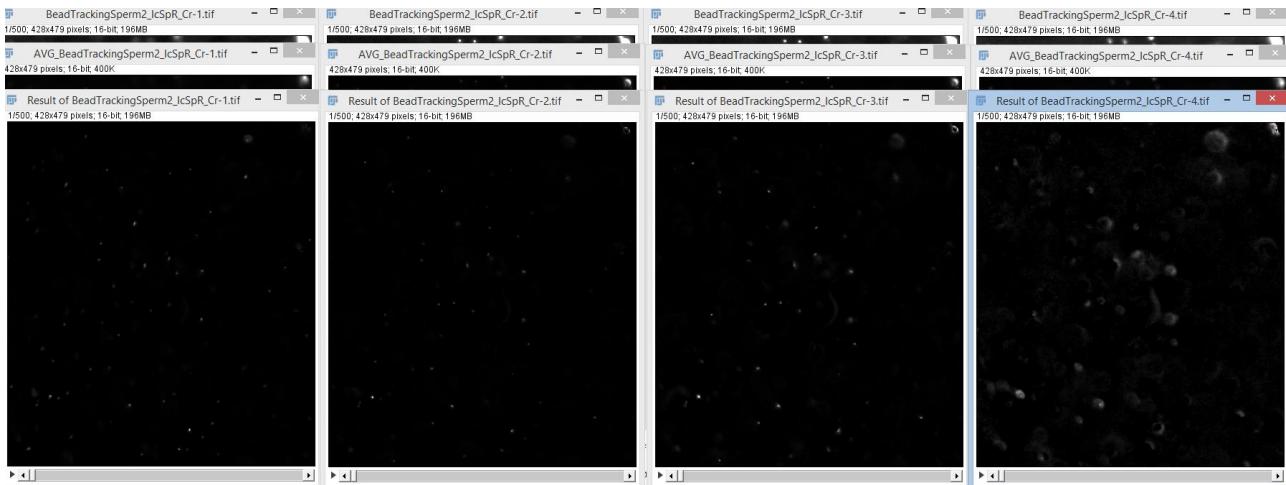


- **Plane 4**

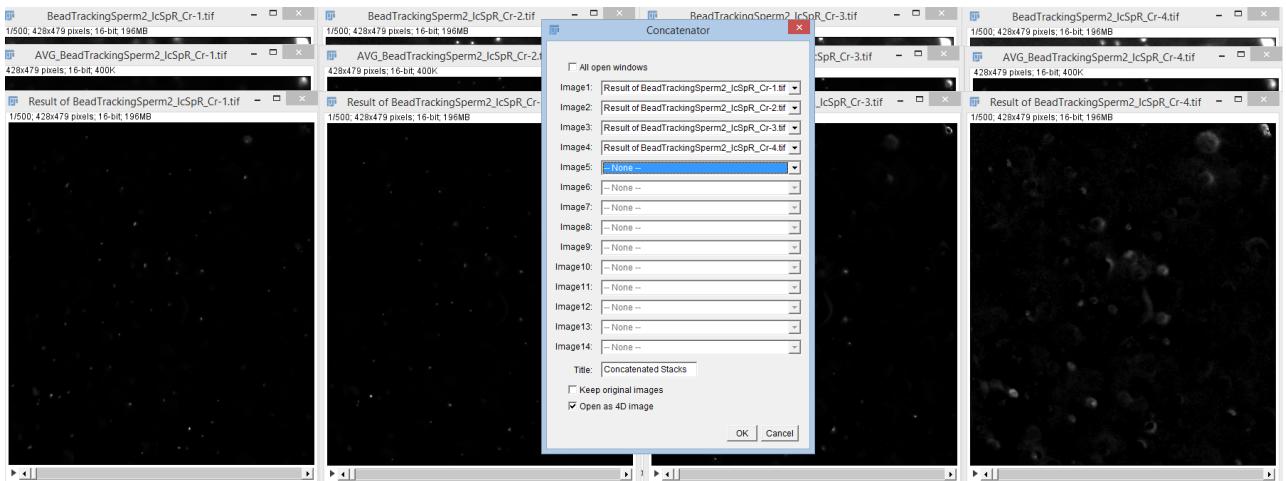


- You should end up with a subtracted image (window title starting with “Result of ...”) for each plane:

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- Merge the “Results of ...” images of all four planes to a hyperstack: Image > Stacks > Tools > Concatenate..., select the four subtracted plane images “Result of ... -1.tif” to “Results of ... -4.tif” as Image1 to Image4, select “-- None --” as Image5, check “Open as 4D image”, and press OK:

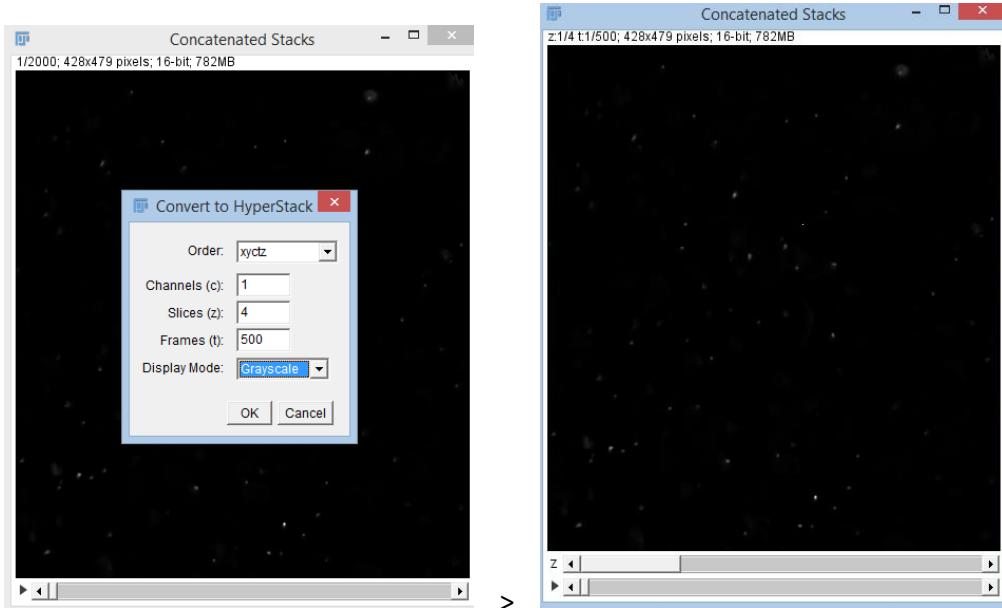


- Eventually the “open as 4D image” function might not work and you will end up with a 1-dimensional stack (the stacks for all four planes are then put into one dimension after each other):

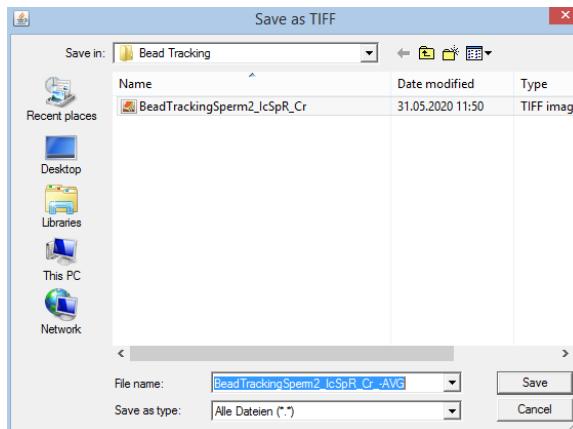


More information, license notes, credits: <https://github.com/hansenjn/MultifocalImaging-AnalysisToolbox>

- If this happens, convert the image to a Multidimensional Hyperstack manually: Image > Hyperstacks > Stack to Hyperstack..., select order “xyctz” and adapt the number of slices and frames according to your image, press OK:



- Save the Concatenated Stacks Image as a .tiff with filename ending “...\_AVG.tif” to note done that the image was corrected for the background: File > Save As... > Tiff...



- Close all open images after the image was saved: Either close each image individually or all at once via File > Close All...

### Determine a calibration Look-Up-Table (LUT)

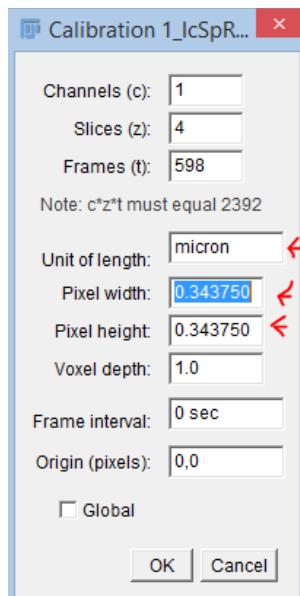
To determine a calibration Look-Up-Table (LUT) you need to record a z-stack with a defined gap between different recorded z-positions, e.g. set by a piezo, through non-moving beads with your multifocal imaging setup. Before performing the steps described in this chapter, make sure the image was preprocessed as described in the previous chapter.

The tools presented in the MultifocalImaging-AnalysisToolbox require to provide a list of particle positions for analysis. This list can be created either manually by noting down the positions of the beads in the image or using the FIJI plugin TrackMate.

- Manually** generating a list of bead positions manually:

More information, license notes, credits: <https://github.com/hansenjn/MultifocalImaging-AnalysisToolbox>

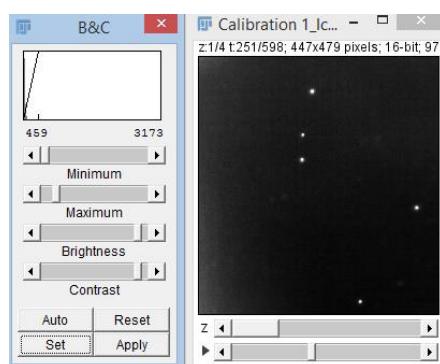
- Create an empty text file with name “<your image’s name>\_spots.txt” and open it so that you can note down the particle positions
- Open the image for which you want to note done particle positions in ImageJ
- Make sure the image is correctly calibrated: Image > Properties; If the information provided at “Unit of length”, “Pixel width”, and “Pixel height” is incorrect, correct it; Press OK.



- Change the stack position with the bars on bottom of the image until you can clearly see the beads you aim to analyze

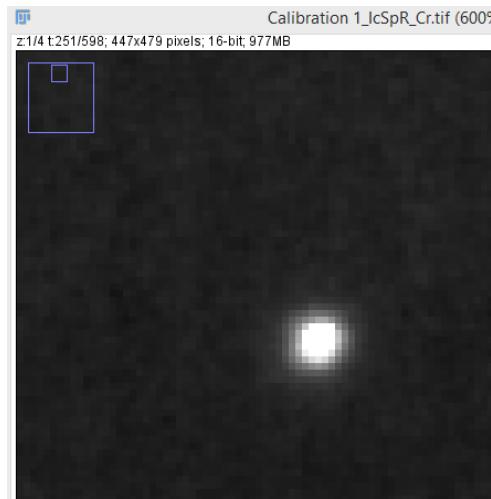


- Eventually you may need to adapt the Brightness and Contrast of the image to see the beads: Image > Adjust > Brightness/Contrast, drag down the maximum

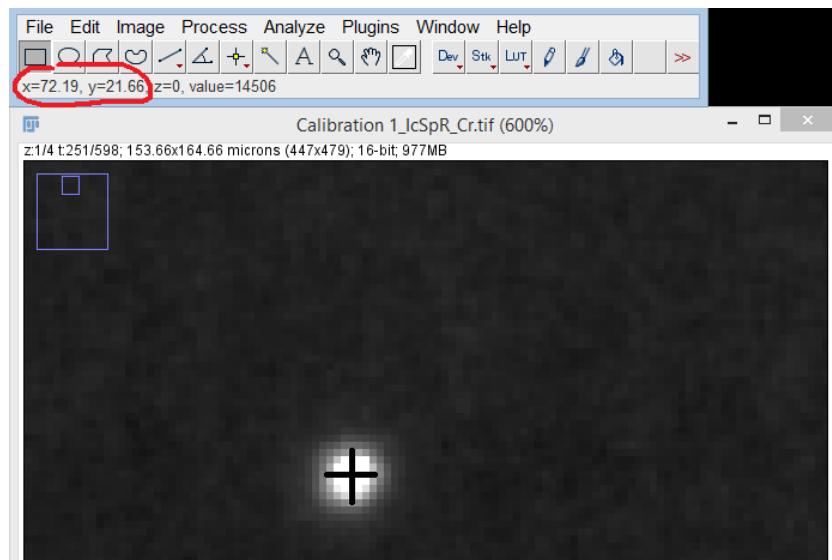


- Zoom into a bead by pressing Ctrl and using the mouse wheel

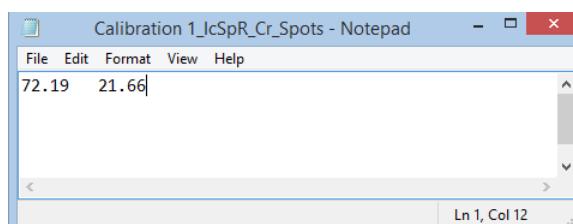
More information, license notes, credits: <https://github.com/hansenjn/MultifocalImaging-AnalysisToolbox>



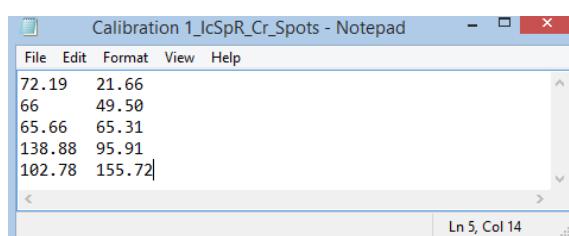
- Hover over the center of a bead to see its calibrated x and y position in the status bar of the ImageJ panel (encircled in red on the image below):



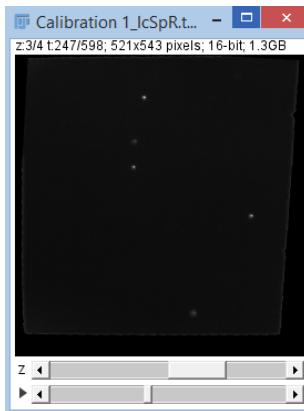
- Note down the coordinates into the text file as <x coordinate>, tab, <y coordinate>



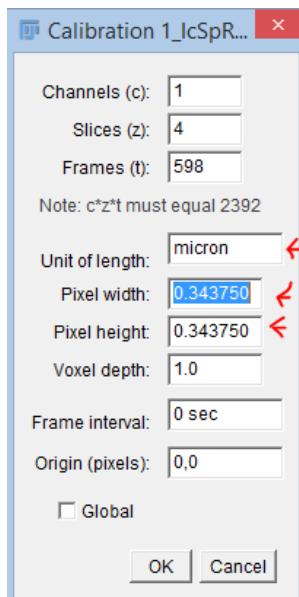
- Zoom into other beads in the image and also note down the coordinates in the text file in additional rows. Each bead position is noted in a separate row.



- After noting down all bead positions, save the text file and close the image.
- Generating a list of bead positions using the FIJI plugin **TrackMate** (Recommended for better precision, reproducibility, and when analyzing many beads):
  - Launch FIJI (this is a special distribution of ImageJ, where the plugin TrackMate is included; <https://imagej.net/Fiji/Downloads>)



- Open the image for which you want to note done particle positions in ImageJ
- Make sure the image is correctly calibrated: Image > Properties; If the information provided at "Unit of length", "Pixel width", and "Pixel height" is incorrect, correct it; Press OK.

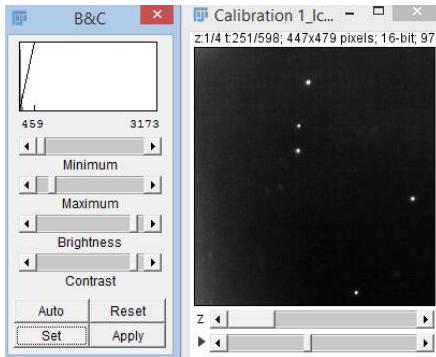


- Change the stack position with the bars on bottom of the image until you can clearly see the beads you aim to analyze

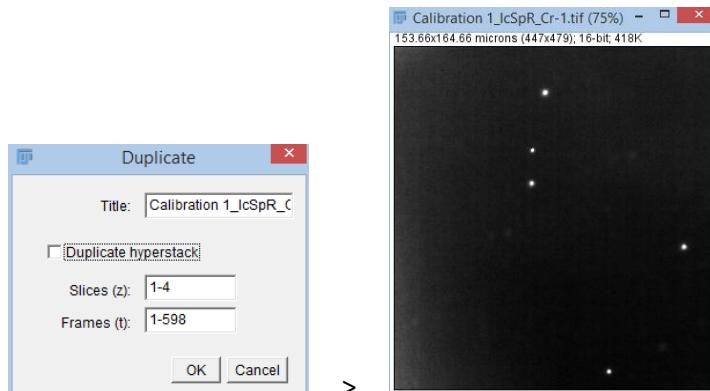


More information, license notes, credits: <https://github.com/hansenjn/MultifocalImaging-AnalysisToolbox>

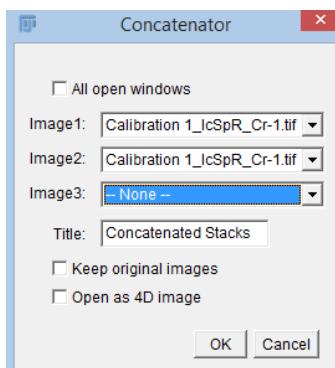
- Eventually you may need to adapt the Brightness and Contrast of the image to see the beads: Image > Adjust > Brightness/Contrast, drag down the maximum



- Duplicate that plane image to use it for analysis by TrackMate: Image > Duplicate, unselect “Duplicate hyperstack”, press OK



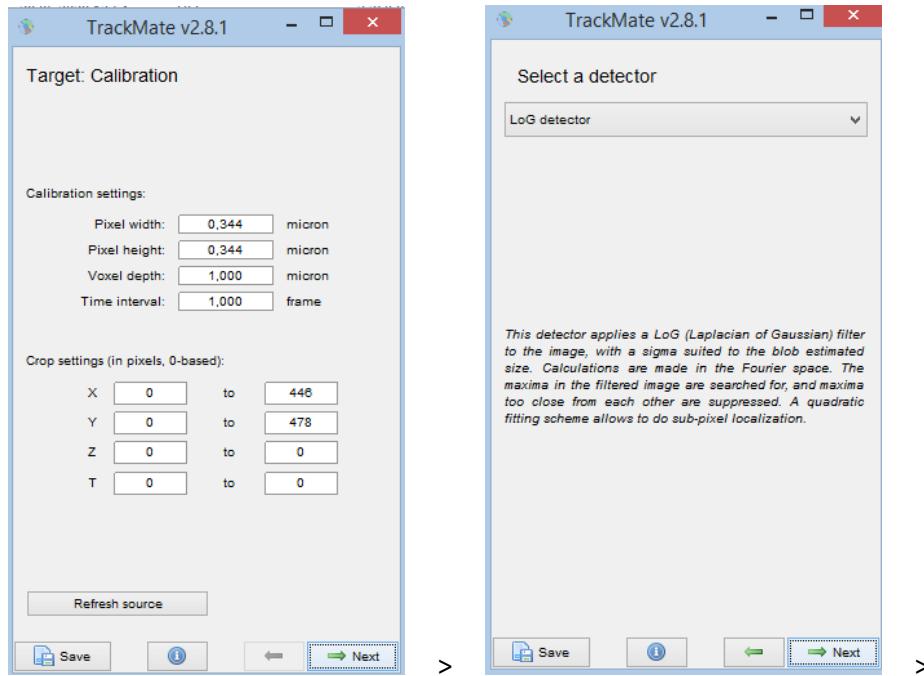
- Create a stack of twice this image: Image > Stacks > Tools > Concatenate..., select the duplicated image as Image1 and as Image2:



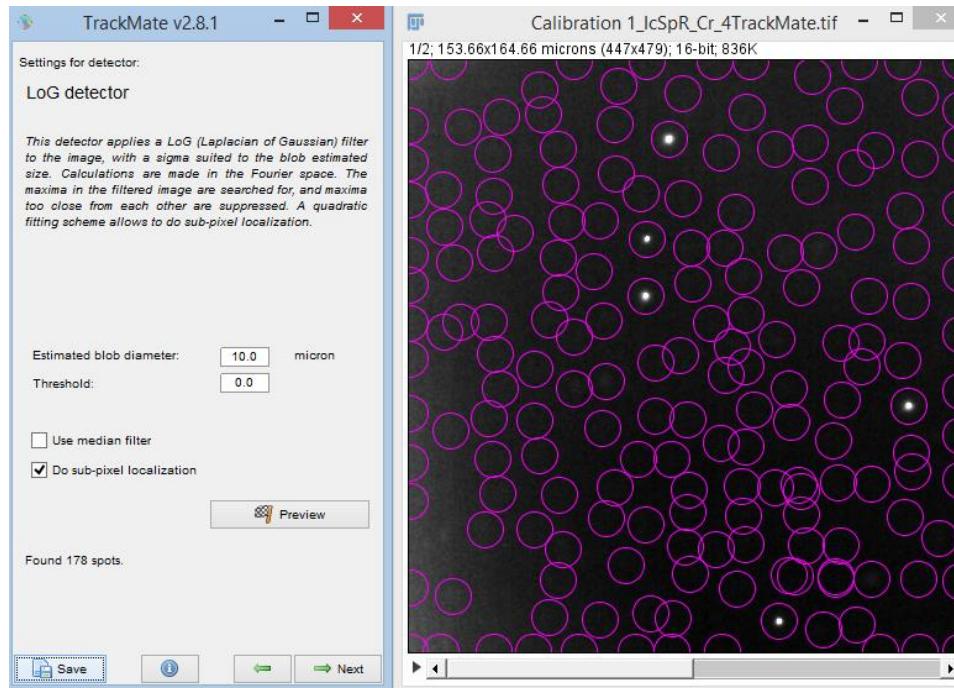
- Save the stack with the ending “\_4TrackMate.tif” to your directory: File > Save As > Tiff...
- Launch TrackMate: Plugins > Tracking > TrackMate; if a dialog pops up asking you to swap Z and T, confirm by pressing Yes (or “Ja” if you have a German computer):



- A dialog pops up - press Next:

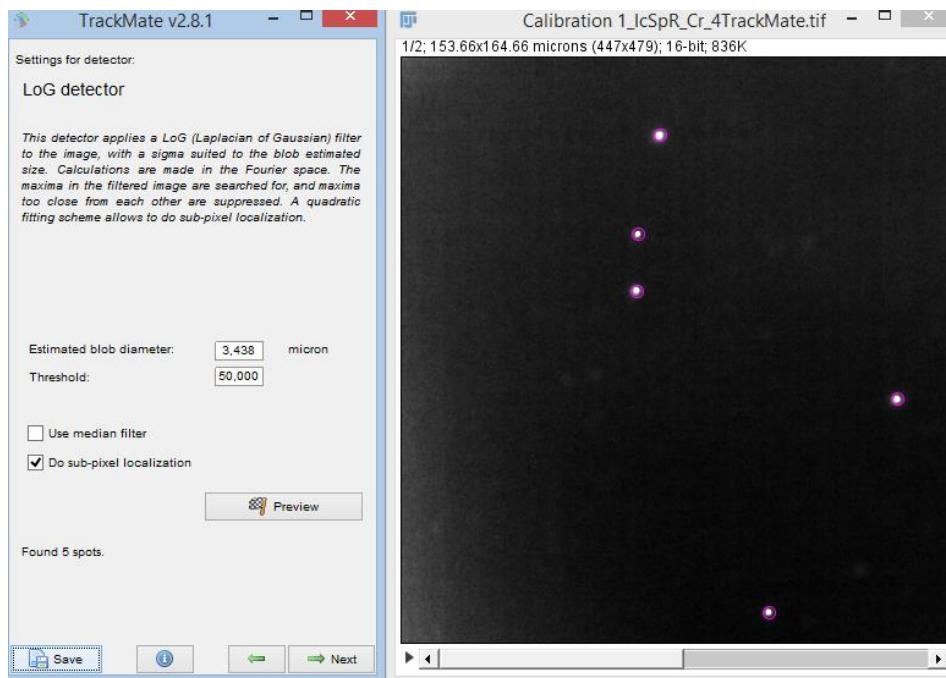


- Press Next and then "Preview" to see the positions that will be detected as a particle (will be encircled in purple)

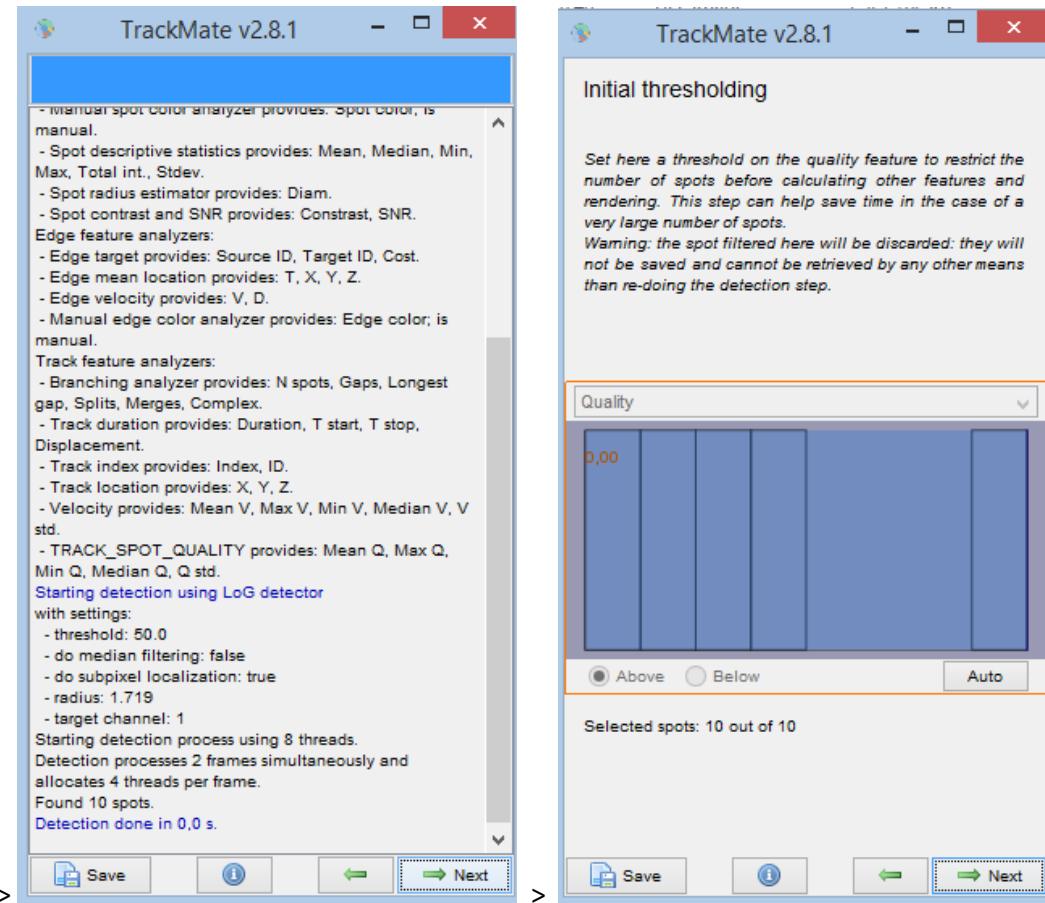


- Adapt the blob diameter and the threshold until the detection is correct.

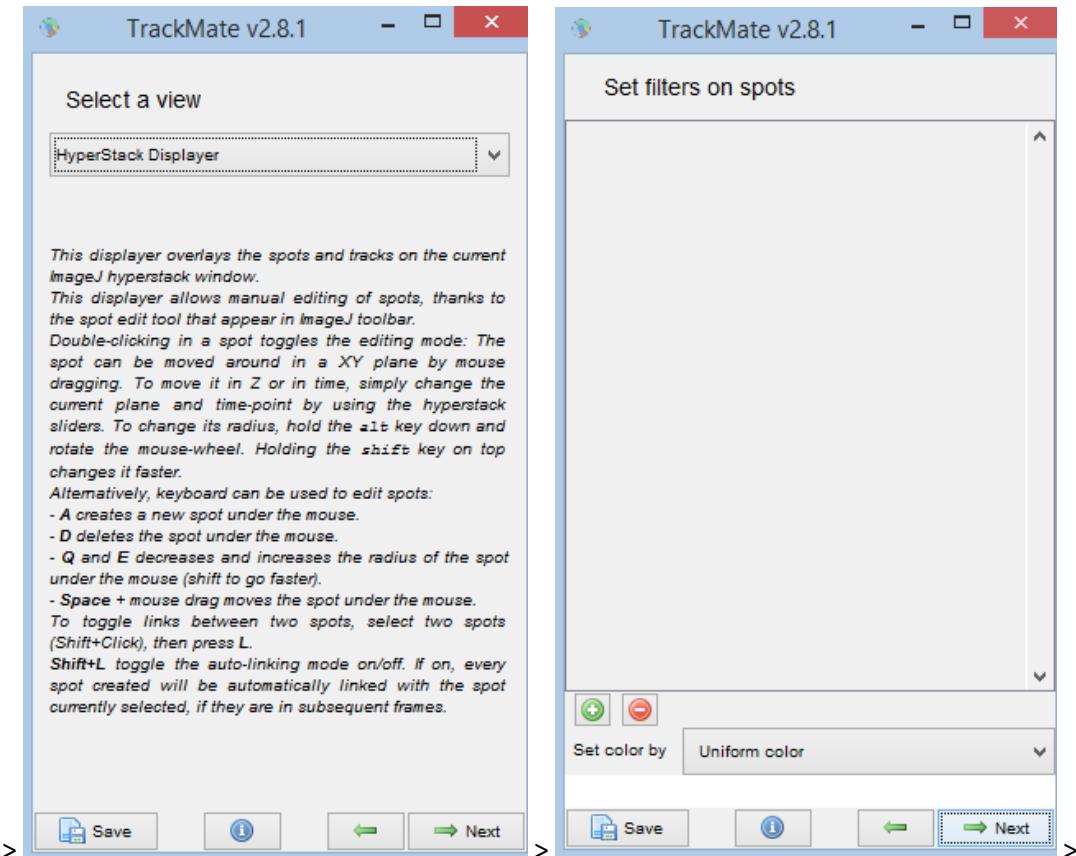
More information, license notes, credits: <https://github.com/hansenjn/MultifocalImaging-AnalysisToolbox>



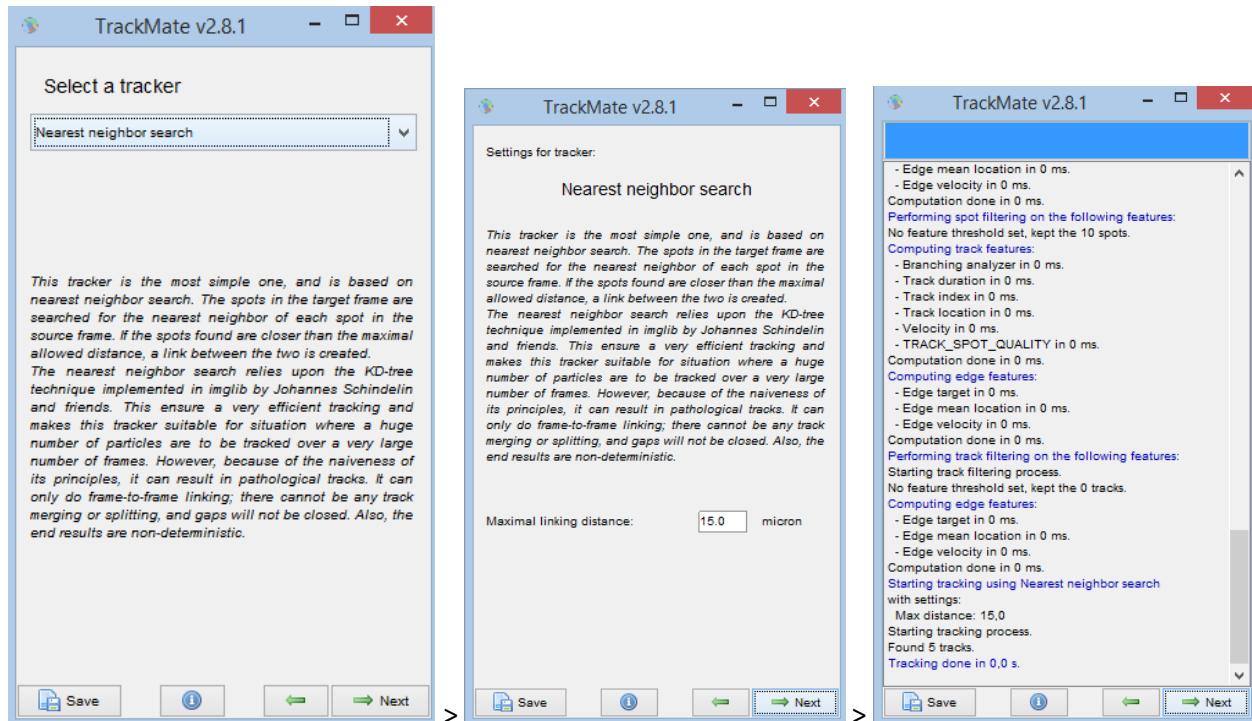
- Click five times Next until you reach a dialog showing “Select Tracker”:

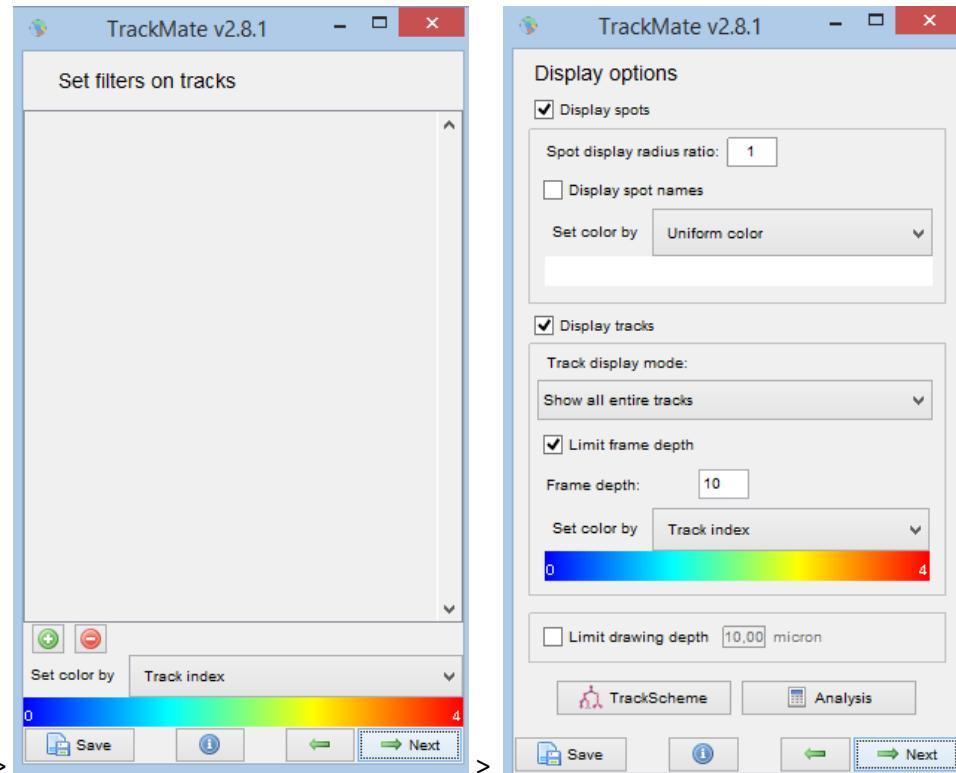


More information, license notes, credits: <https://github.com/hansenjn/MultifocalImaging-AnalysisToolbox>



- In the dialog “Select a tracker”, select “Nearest neighbor search” and press Next for four times:

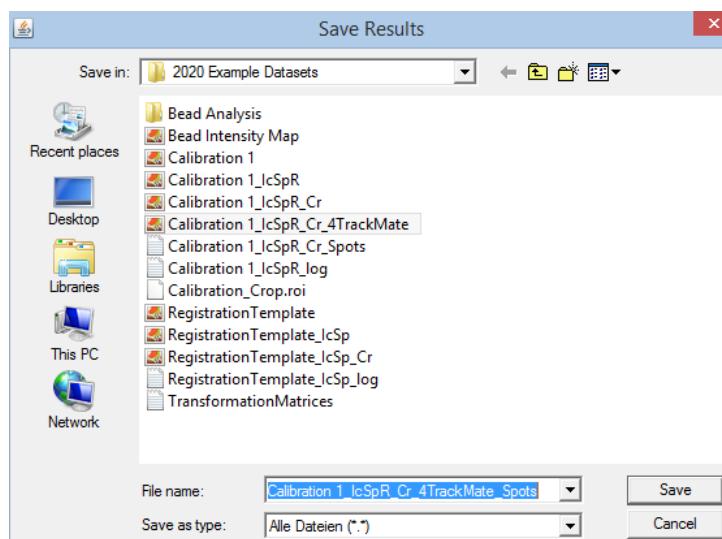




- Press “Analysis” and three windows pop up:

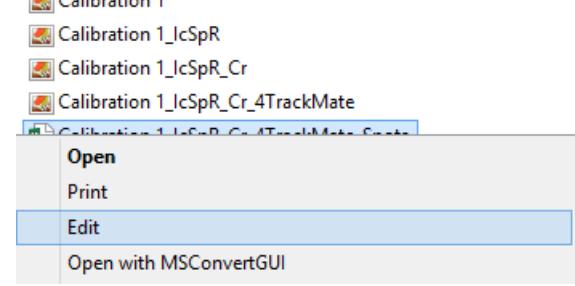
Track statistics				Links in tracks statistics				Spots in tracks statistics				
Label	NUMBER_SPOTS	NUMBER_GAPS	LONGEST	Label	TRACK_ID	SPOT_SOURCE_ID	SPOT	Label	ID	TRACK_ID	QUALITY	POSITION
1 Track_0	2	0	0	1 (ID387 : ID389)	0	387	389	1 ID387	387	0	460.24277	72.24119
2 Track_1	2	0	0	2 (ID386 : ID390)	1	386	390	2 ID389	389	0	460.24277	72.24119
3 Track_2	2	0	0	3 (ID385 : ID393)	2	385	393	3 ID386	386	1	396.92221	66.12997
4 Track_3	2	0	0	4 (ID384 : ID392)	3	384	392	4 ID390	390	1	396.92221	66.12997
5 Track_4	2	0	0	5 (ID388 : ID391)	4	388	391	5 ID385	385	2	425.63721	138.99866

- Close the windows “Track statistics” and “Links in tracks statistics” without saving, select the window “Spots in tracks statistics” and save it as a .csv file: File > Save As ...

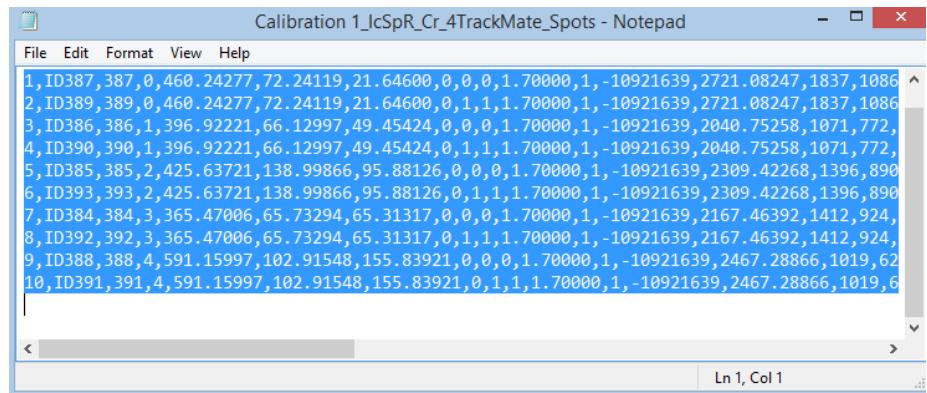


- Close all images and windows in Fiji.
- Extract the points from the .csv file and save them into a text file (one row per position, in each row: <x-position> tab <y-position>). This can be done manually using a table-calculation-software like Excel or programmatically in MatLab or R by importing the .csv file, extracting the X and Y positions (“Position\_X”, “Position\_Y” in the file) for the timepoint (“Position\_T”) 0, and saving them automatically into a text file. See here an exemplary way to extract the positions in Windows and using Excel:

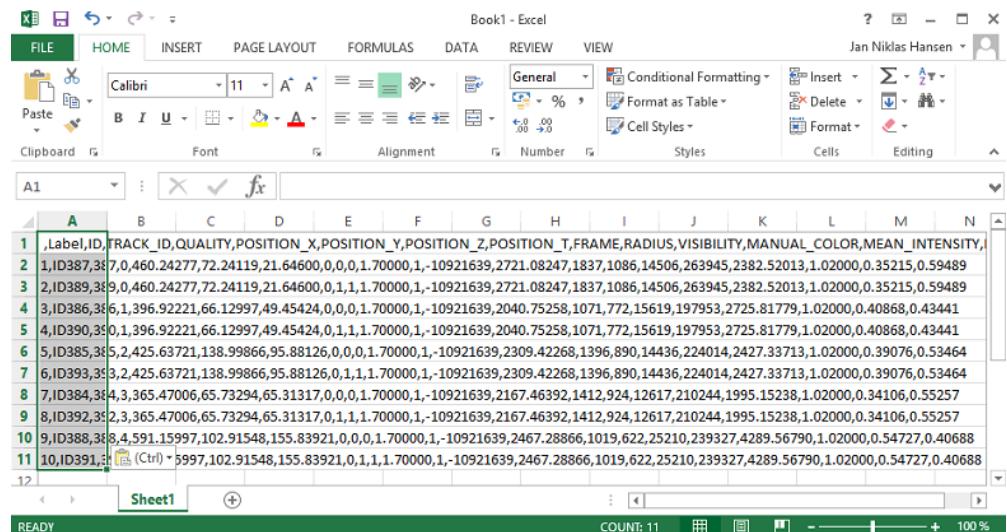
- Open the saved .csv file with a text editor



- Mark all and press Ctrl + C:

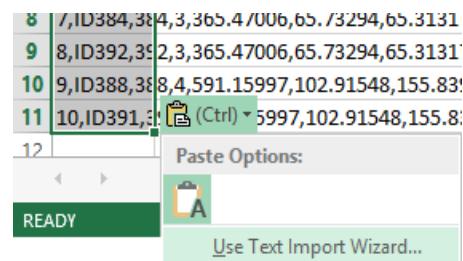


- Open Excel or a similar table calculation software and press Ctrl + V

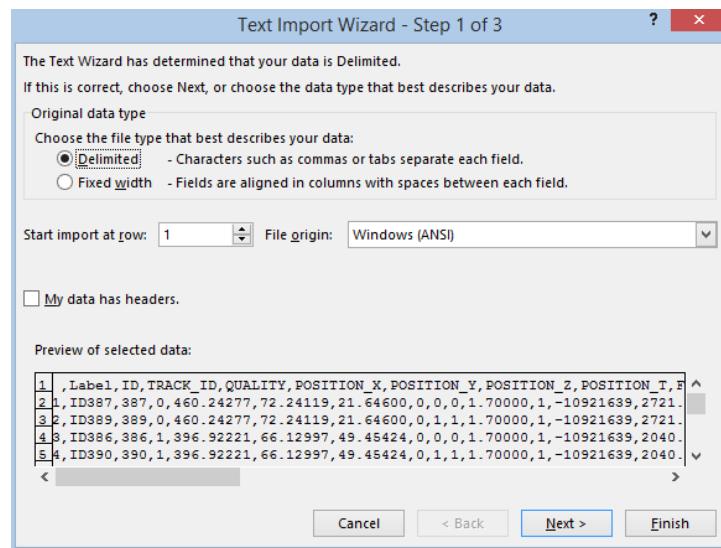


- Click on the small Ctrl button on the bottom and select “Use Text Import Wizard...”

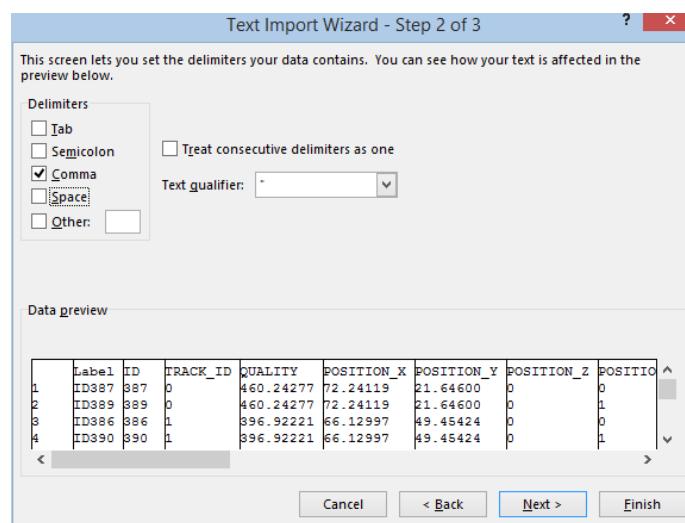
More information, license notes, credits: <https://github.com/hansenjn/MultifocalImaging-AnalysisToolbox>



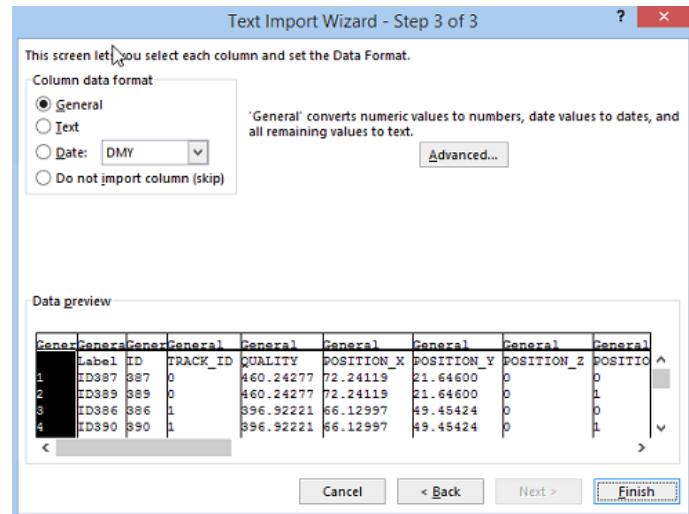
- Select Delimited and press Next



- Select Comma (see below) and press Next



- Press Finish



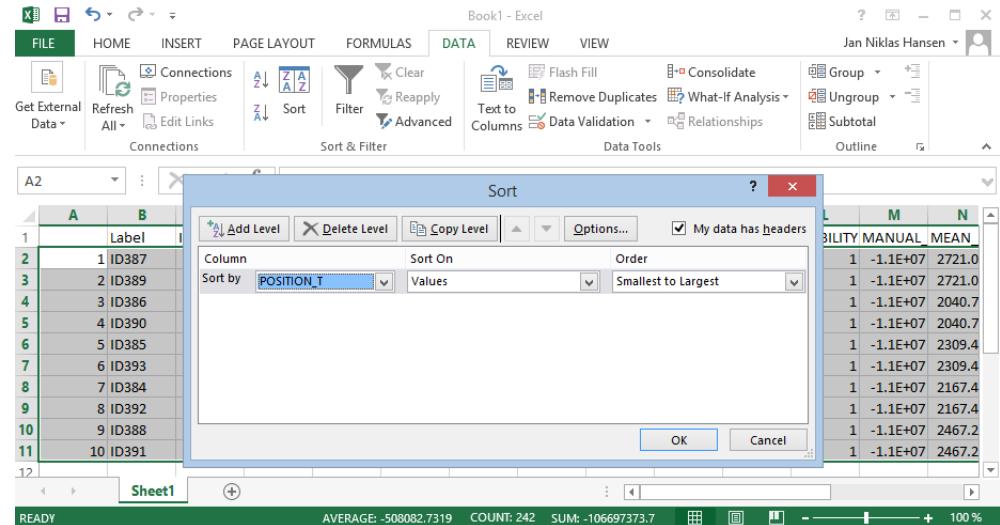
- Select all (usually all is already selected)

A	B	C	D	E	F	G	H	I	J	K	L	M	N
1	Label	ID	TRACK_ID	QUALITY	POSITION_X	POSITION_Y	POSITION_Z	POSITION_T	FRAME	RADIUS	VISIBILITY	MANUAL_MEAN	
2	1	ID387	387	0	460.2428	72.24119	21.646	0	0	0	1.7	1	-1.1E+07 2721.0
3	2	ID389	389	0	460.2428	72.24119	21.646	0	1	1	1.7	1	-1.1E+07 2721.0
4	3	ID386	386	1	396.9222	66.12997	49.45424	0	0	0	1.7	1	-1.1E+07 2040.7
5	4	ID390	390	1	396.9222	66.12997	49.45424	0	1	1	1.7	1	-1.1E+07 2040.7
6	5	ID385	385	2	425.6372	138.9987	95.88126	0	0	0	1.7	1	-1.1E+07 2309.4
7	6	ID393	393	2	425.6372	138.9987	95.88126	0	1	1	1.7	1	-1.1E+07 2309.4
8	7	ID384	384	3	365.4701	65.73294	65.31317	0	0	0	1.7	1	-1.1E+07 2167.4
9	8	ID392	392	3	365.4701	65.73294	65.31317	0	1	1	1.7	1	-1.1E+07 2167.4
10	9	ID388	388	4	591.16	102.9155	155.8392	0	0	0	1.7	1	-1.1E+07 2467.2
11	10	ID391	391	4	591.16	102.9155	155.8392	0	1	1	1.7	1	-1.1E+07 2467.2

- Sort the data: DATA > Sort

A	B	C	TRA	POSITION_X	POSITION_Y	POSITION_Z	POSITION_T	FRAME	RADIUS	VISIBILITY	MANUAL_MEAN	
1	Label	ID	TRACK_ID	21.646	0	0	0	0	1.7	1	-1.1E+07	2721.0
2	1	ID387	387	0	460.2428	72.24119	21.646	0	1	1	1.7	1
3	2	ID389	389	0	460.2428	72.24119	21.646	0	1	1	1.7	1
4	3	ID386	386	1	396.9222	66.12997	49.45424	0	0	0	1.7	1
5	4	ID390	390	1	396.9222	66.12997	49.45424	0	1	1	1.7	1
6	5	ID385	385	2	425.6372	138.9987	95.88126	0	0	0	1.7	1
7	6	ID393	393	2	425.6372	138.9987	95.88126	0	1	1	1.7	1
8	7	ID384	384	3	365.4701	65.73294	65.31317	0	0	0	1.7	1
9	8	ID392	392	3	365.4701	65.73294	65.31317	0	1	1	1.7	1
10	9	ID388	388	4	591.16	102.9155	155.8392	0	0	0	1.7	1
11	10	ID391	391	4	591.16	102.9155	155.8392	0	1	1	1.7	1

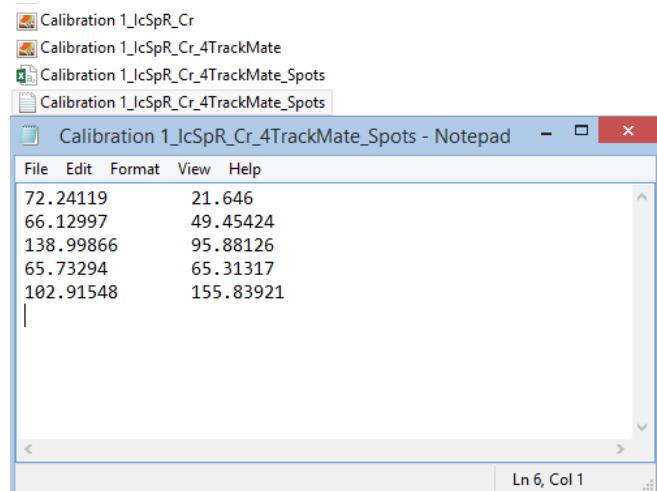
- Select POSITION\_T as "Sort by" and press OK:



- Select the X and Y Coordinates belonging to a “POSITION\_T” of 0 and copy them by pressing Ctrl + C

D	QUALITY	POSITION_X	POSITION_Y	POSITION_Z	POSITION_T
0	460.2428	72.24119	21.646	0	0
1	396.9222	66.12997	49.45424	0	0
2	425.6372	138.99866	95.88126	0	0
3	365.4701	65.73294	65.31317	0	0
4	591.16	102.91548	155.83921	0	0
0	460.2428	72.24119	21.646	0	1
1	396.9222	66.12997	49.45424	0	1
2	425.6372	138.99866	95.88126	0	1
3	365.4701	65.73294	65.31317	0	1
4	591.16	102.91548	155.83921	0	1

- Create a text file in the directory of the image and name it “<image name>\_4TrackMate\_Spots.txt”, open it, and paste the coordinates by pressing Ctrl + V, save it, and close it:

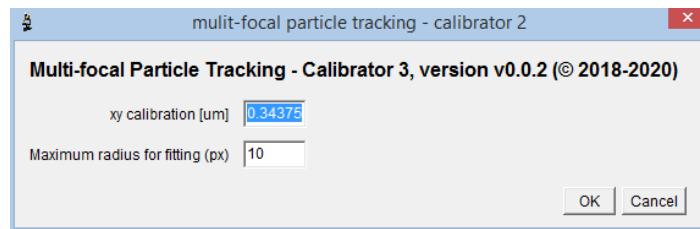


- Save the text file.

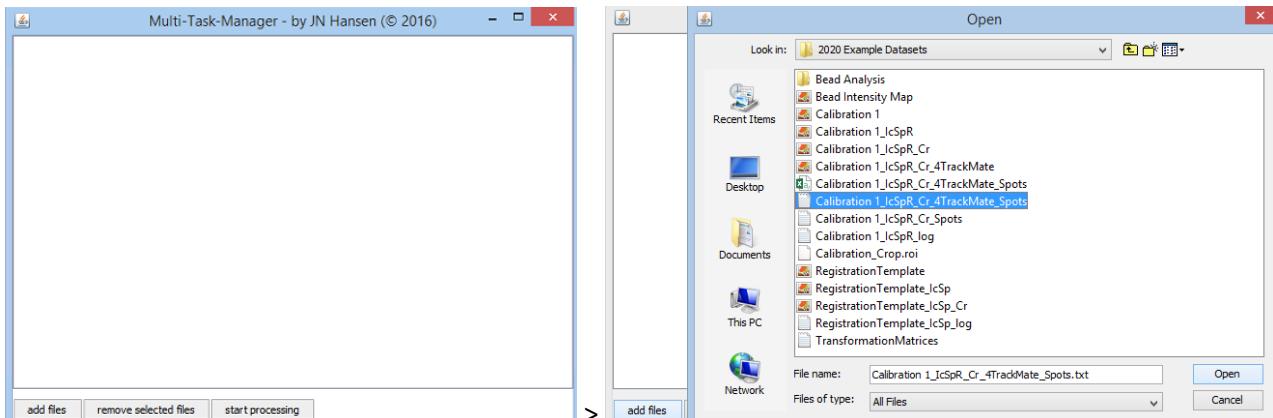
Now that a file that contains the positions to be analyzed has been created, analysis of the bead width at different z positions with the MultiFocalParticleTracker-Calibrator plugin can be conducted:

More information, license notes, credits: <https://github.com/hansenjn/MultifocalImaging-AnalysisToolbox>

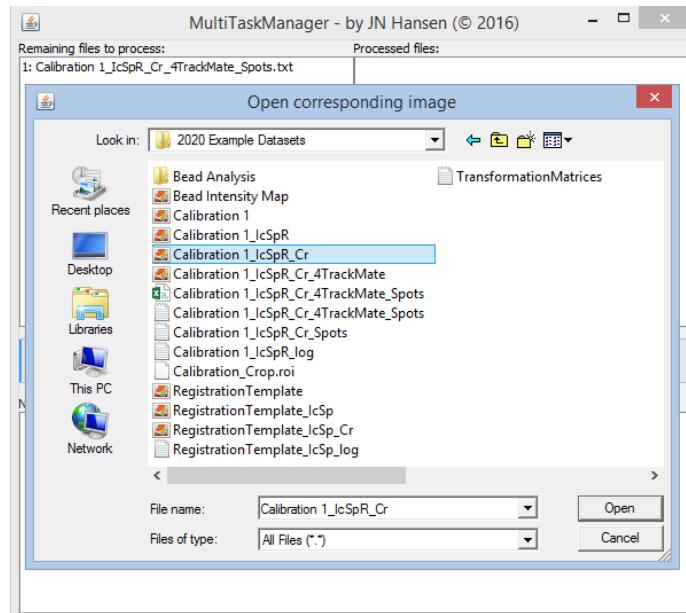
- Open Image
- Install the latest release of the plugin MultiFocalParticleTracker-Calibrator-3: <https://github.com/hansenjn/MultiFocalParticleTracker-Calibrator-3/releases/>
- Restart ImageJ
- Launch MultiFocalParticleTracker-Calibrator-3: Plugins > JNH > Multi Focal > Calibrate Particles 3 circ ...
- Enter the settings according to your analysis
  - Enter the xy calibration of your image – for the exemplary data set: 0.34375  $\mu\text{m}$  / px
  - Enter the radius that you want to consider for estimating the width of the bead.
    - If the radius is small beads can detected only across a small depth
    - If the radius is too big beads can be detected across a high depth but the width estimation might get incorrect when other beads are also present within that radius
    - Note: the same radius needs to be selected later on in *MultiFocalParticleTracking – Complex 3*, otherwise the inference of z-positions might be incorrect.
    - For the exemplary data set: 10 px



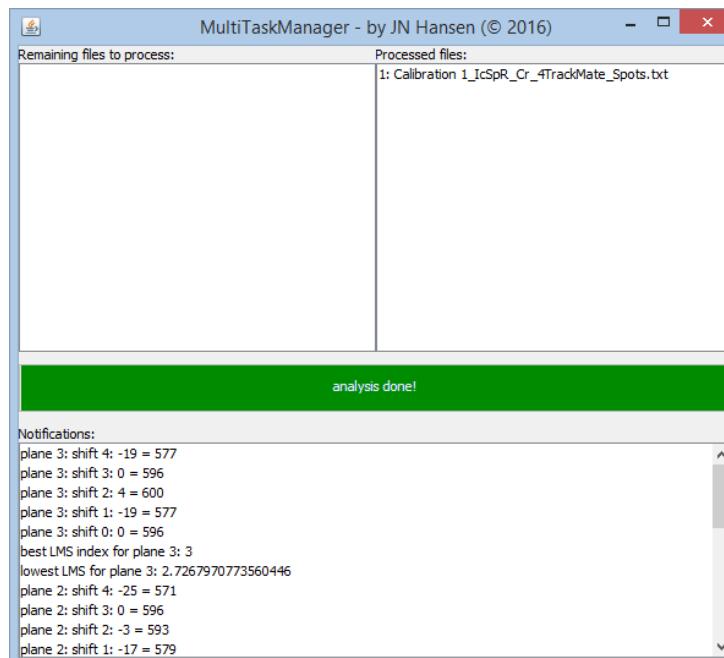
- A dialog pops up: add the text file containing the bead positions and press “start processing”:



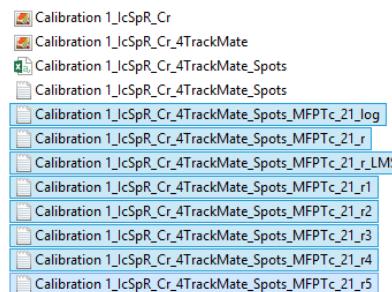
- A dialog pops up requesting to open the corresponding image. Select the corresponding image and press Open:



- Wait until MultiTaskManager states “analysis done!”

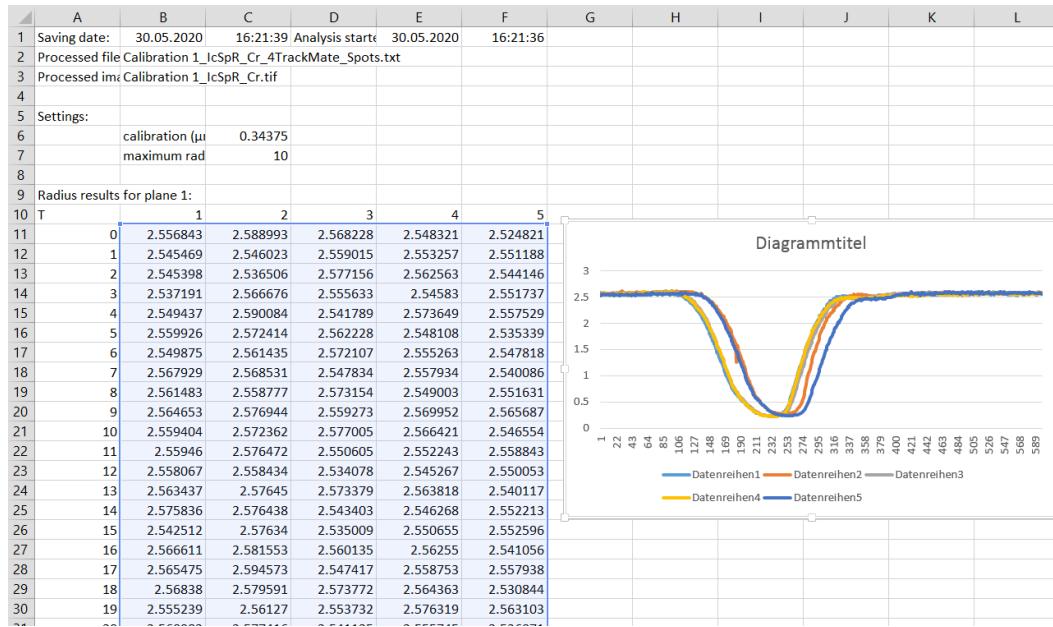


- The plugin has saved new files to the folder where the text file with bead position was saved. They all contain an additional suffix (“\_MFPTc\_<number>\_” ) and specific file endings.



More information, license notes, credits: <https://github.com/hansenjn/MultifocalImaging-AnalysisToolbox>

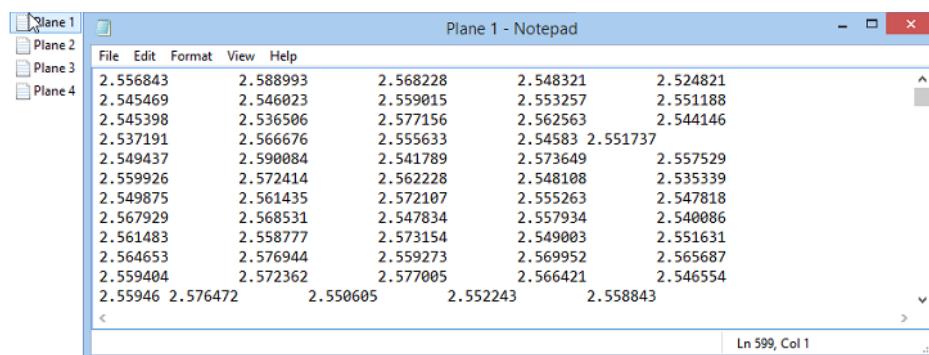
- Open the file with ending "...\_r.txt" and copy the content e.g. to excel to investigate the determined widths:



- You find for each plane a table with width values (scroll down to find tables for the other planes).
  - If you have analyzed a stack where the beads largely defocus, you may see that the width saturates at a distinct value (e.g. 2.5) depending on which radius you have set in the plugin for processing. This happens when the bead gets so defocused that the width exceeds the circular area that is used to determine the width.
  - Usually the beads are not located on the same heights, thus the curves are shifted.

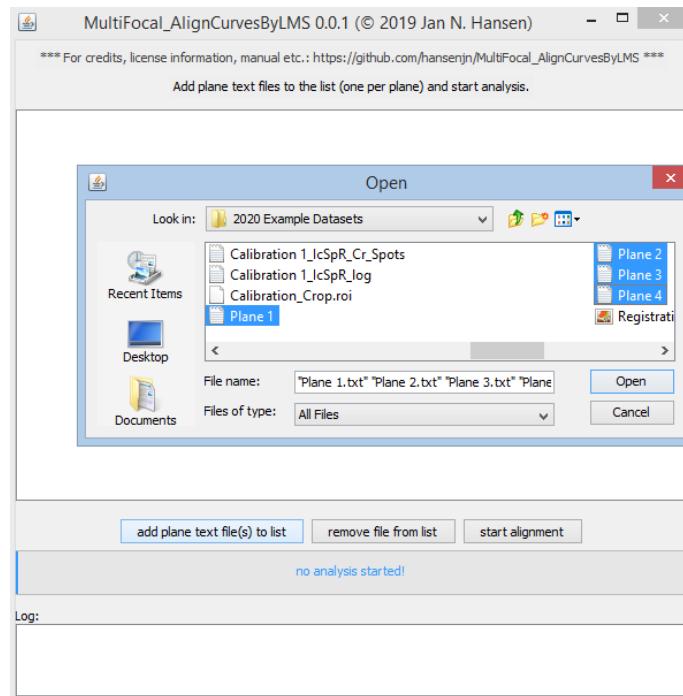
Align the Curves by minimizing the least-mean-square (LMS) of the differences between beads:

- Generate 4 text files, entitled e.g.:
  - Plane 1.txt
  - Plane 2.txt
  - Plane 3.txt
  - Plane 4.txt
- Copy the pure width values as selected in the image above for each plane to the corresponding text file, e.g.

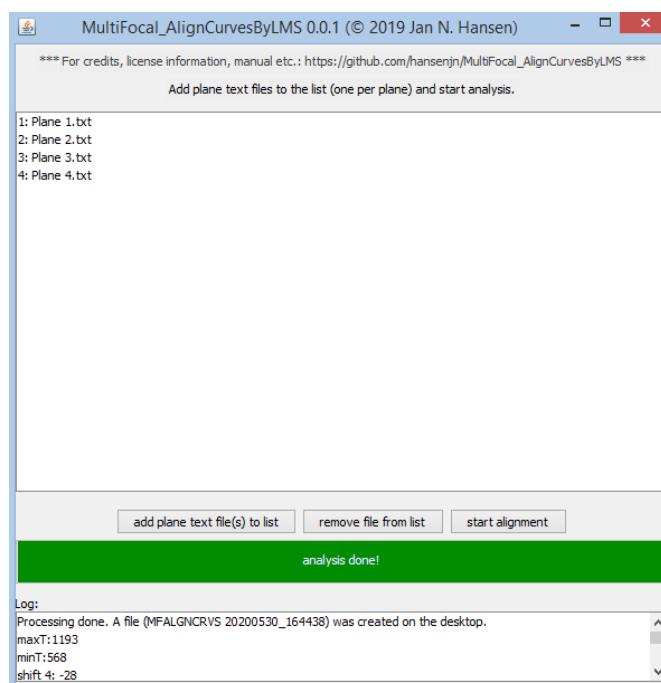


- In each file, each column must contain the width values for one bead at different z-positions
  - Do not include a column with slice positions!
  - Do not include a first row with bead labels!

- You may add data from other images to additional columns in the file
- When you have copied the width values for each plane to the corresponding text file, launch the java application MultiFocal\_AlignCurvesByLMS (can be downloaded at: [https://github.com/hansenjn/MultiFocal\\_AlignCurvesByLMS/releases](https://github.com/hansenjn/MultiFocal_AlignCurvesByLMS/releases)).
- Add the plane text files to the list: press “add plane text file(s)”, select the text files, press Open.

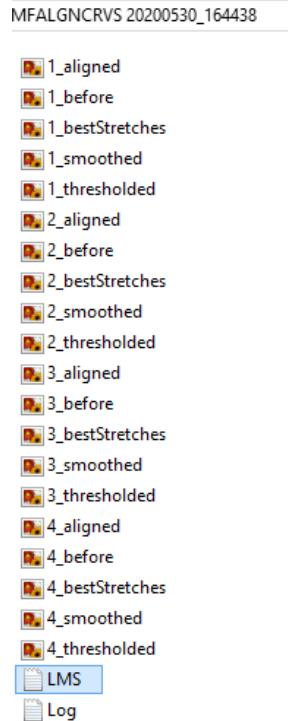


- Press start alignment and wait until analysis is done. During analysis, the plugin shifts the curves of the beads along each other (but always with the same shift in all planes) and determines the LMS between the curves across all planes. The shift with the minimum LMS result is selected and a table is output where the individual bead curves are shifted accordingly.

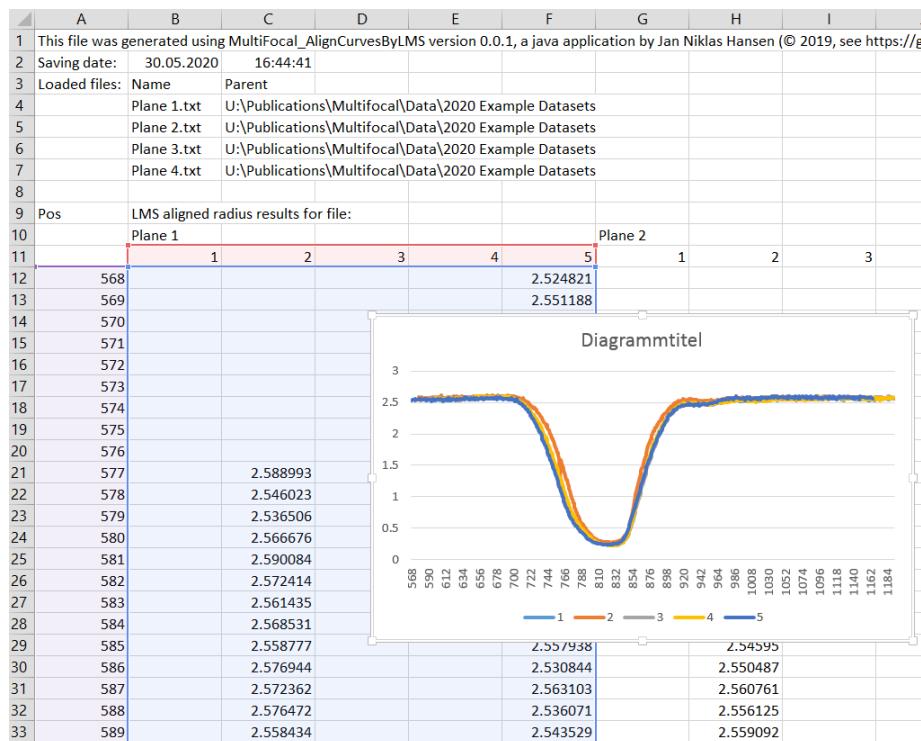


More information, license notes, credits: <https://github.com/hansenjn/MultifocalImaging-AnalysisToolbox>

- As written in the Log window, on the desktop / in the home directory a folder has been created that contains the results from curve alignment. The folder name starts with “MFALGNCRVS...”.



- Open the LMS.txt file and investigate the output table, e.g. copy it to excel:



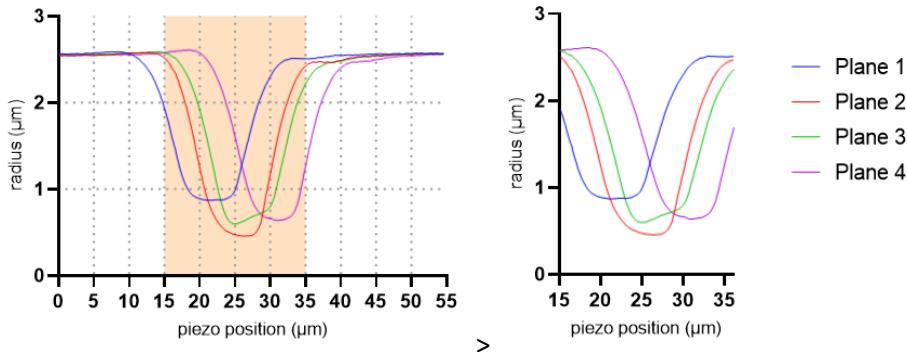
Now the curves have been aligned and the aligned data can be used to create a calibration Look-Up-Table (LUT) as follows:

- For each plane, average the different widths values at each position

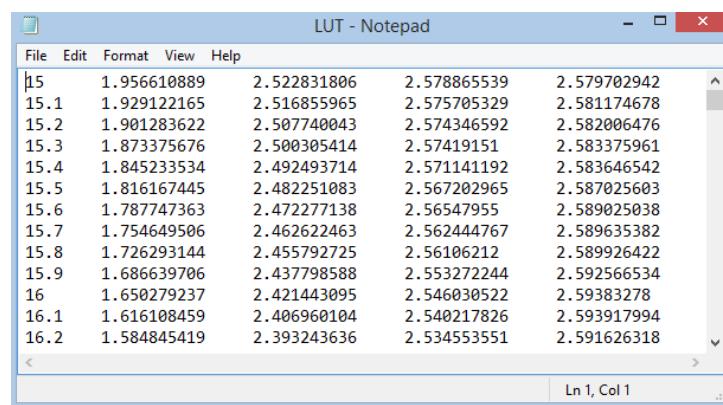
- Thereby, create a table indicating the z-position and the four columns (one for each plane)

	A	B	C	D	E	I
1	z	Plane 1	Plane 2	Plane 3	Plane 4	
2	0.0	2.524821	2.541617	2.532908	2.560373	
3	0.1	2.551188	2.549706	2.526473	2.57322	
4	0.2	2.544146	2.534016	2.53904	2.572483	
5	0.3	2.551737	2.552211	2.539125	2.558687	
6	0.4	2.557529	2.529922	2.56674	2.566806	
7	0.5	2.535339	2.548054	2.524515	2.567182	
8	0.6	2.547818	2.539055	2.545332	2.554406	
9	0.7	2.540086	2.549106	2.551221	2.611402	
10	0.8	2.551631	2.536646	2.5731	2.569227	
11	0.9	2.57734	2.552787	2.559608	2.5742	
12	1.0	2.546289	2.549	2.557591	2.567861	
13	1.1	2.547675	2.544107	2.549	2.567757	
14	1.2	2.558365	2.553404	2.561782	2.557805	
15	1.3	2.565101	2.552527	2.534135	2.567829	
16	1.4	2.562314	2.556816	2.544481	2.569343	
17	1.5	2.557016	2.547396	2.543502	2.558728	
18	1.6	2.554794	2.546856	2.557332	2.553767	
19	1.7	2.558358	2.546064	2.542272	2.563847	
20	1.8	2.553894	2.560146	2.545436	2.562794	
21	1.9	2.567722	2.554887	2.544516	2.5612825	

- Eventually, use a smoothing algorithm to remove noise
- Plot the data and select a region of z-positions, in which at any z-position at least one plane features width values that are not in saturation. Then reduce the table to that range:



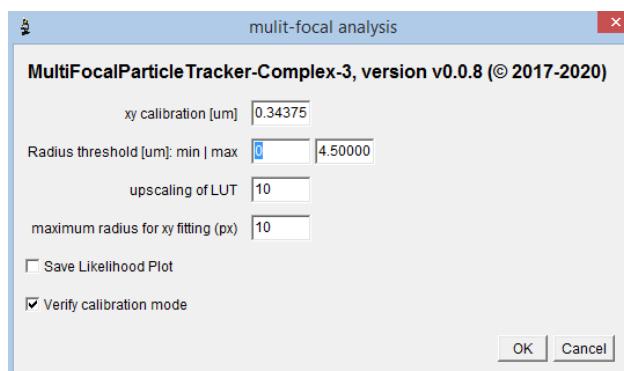
- Copy the table range into a text-file and save it as LUT.txt
  - Do not include a head / first row with information about the planes
  - The first column represents the z-position, the second column represents the width in plane image 1, the third column represents the width in plane image 2, etc.



## Verify the calibration Look-Up-Table (LUT)

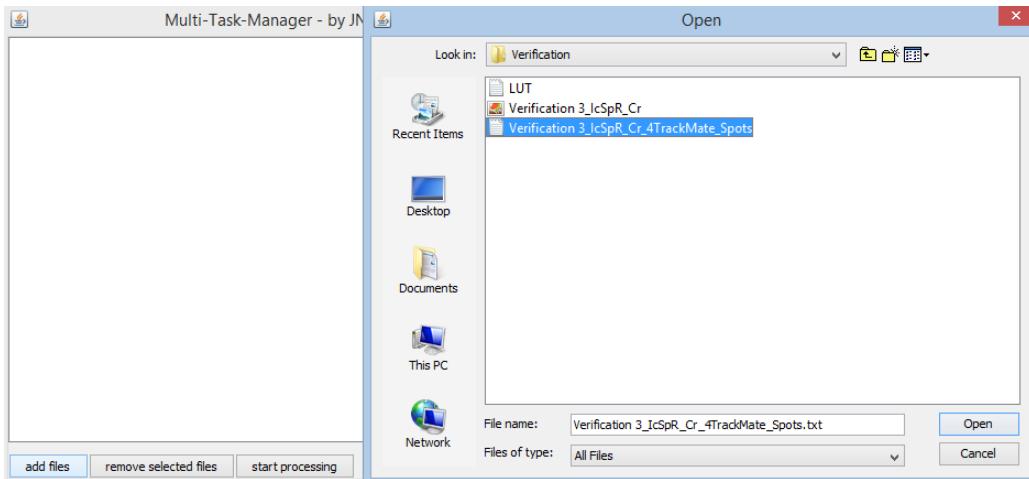
To verify the LUT you use an image as generated for recording the LUT. However, this image should not be used for the LUT to assure that you can apply the LUT also to different individual beads than those that have been used to generate the LUT. Process the image as described in the beginning of the chapter “Determine a calibration Look-Up-Table (LUT)”: preprocess the image, check the image calibration, generate a text file containing the bead positions. Then, proceed as follows.

- Open ImageJ
- Install the latest release of the plugin MultiFocalParticleTracker-Complex-3: <https://github.com/hansenjn/MultiFocalParticleTracker-Complex-3/releases/>
- Restart ImageJ
- Launch MultiFocalParticleTracker-Complex-3: Plugins > JNH > Multi Focal > Complex Particle Tracking 3...
- Enter the settings according to your analysis
  - Xy calibration [μm]: enter the xy calibration of your image – for the exemplary data set: 0.34375 μm / px
  - Radius threshold [um] ...: allows to exclude determined width values below the min and above the max threshold from including them into calculating the z-position. If no threshold shall be used – as in the exemplary analysis – set the min to 0 and the max to a value above the highest value in your LUT.
  - Upscaling of LUT: To obtain a more precise z estimation, a spline interpolation can be used to add intermediate steps between the z-position-width-values indicated in the LUT. E.g. when setting the upscaling value to 10, between two lines in the LUT, 9 more lines are added with equidistant steps. The width values for these extra steps are determined by spline interpolation from the neighbored widths values.
  - Maximum radius for xy fitting: Enter the radius that you have entered in MultiFocalParticleTracker-Calibration-3 during LUT generation: for the exemplary data set: 10 px
  - Save Likelihood Plot: do not check / ignore
  - Verify calibration mode: check to perform the verification

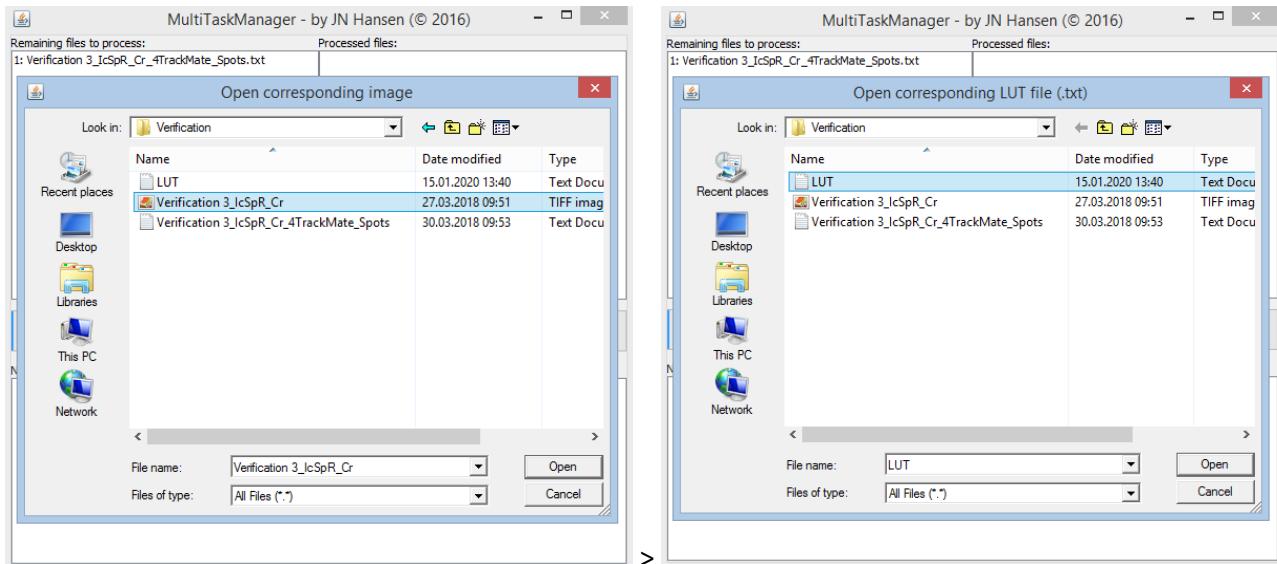


- A dialog pops up: add the text file containing the bead positions and press “start processing”:

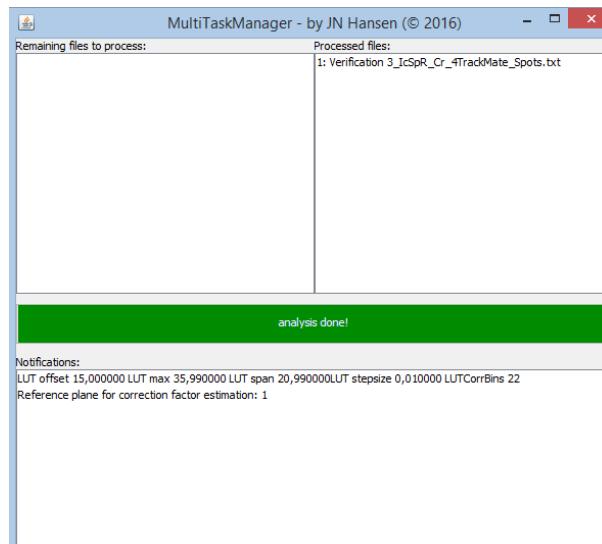
More information, license notes, credits: <https://github.com/hansenjn/MultifocalImaging-AnalysisToolbox>



- A dialog pops up requesting to open the corresponding image. Select the corresponding image and press Open. Next, dialog pops up requesting you to load the LUT. Select the LUT and press Open.

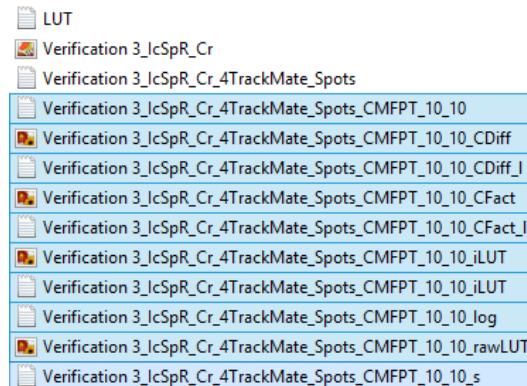


- Wait until MultiTaskManager states “analysis done!”

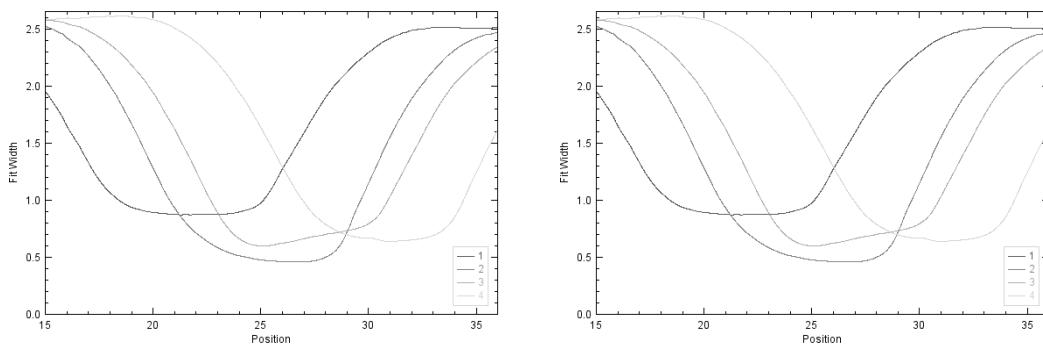


More information, license notes, credits: <https://github.com/hansenjn/MultifocalImaging-AnalysisToolbox>

- The plugin has saved new files to the folder where the text file with bead position was saved. They all contain an additional suffix ("\_CMFPT\_<numbers>\_") and specific file endings.



- ...rawLUT.png shows a plot of the LUT before and ...iLUT.png after spline interpolation:



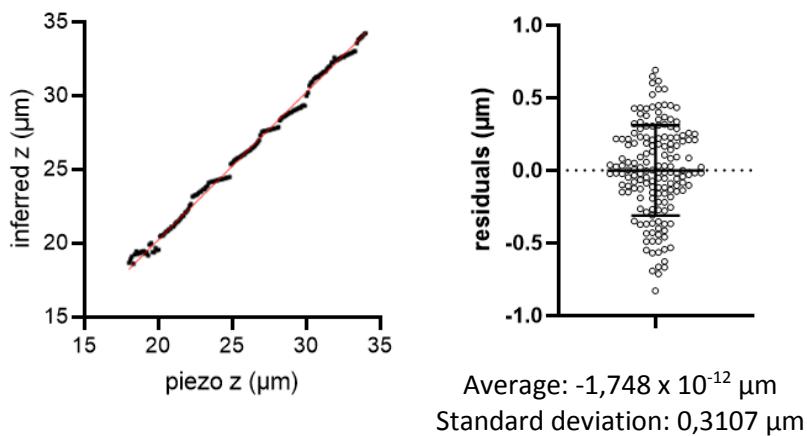
- Open the file with ending "...\_s.txt" and copy the content e.g. to excel to investigate the results.

File	Edit	Format	View	Help	30.05.2020 23:18	Text Document
Verification 3_lcSpR_Cr_4TrackMate_Spots_CMFPT_10_10_s - Notepad						X
index	T	X (μm)	Y (μm)	Z average (μm)	factor-corr.	Z avg (μm)
0	0,000000	75,678969	58,700125	35,093333		
1	1,000000	75,678969	58,700125	34,645000		
2	2,000000	75,678969	58,700125	35,093333		
3	3,000000	75,678969	58,700125	35,093333		
4	4,000000	75,678969	58,700125	35,093333		
5	5,000000	75,678969	58,700125	35,317500		
6	6,000000	75,678969	58,700125	35,093333		
7	7,000000	75,678969	58,700125	35,093333		
8	8,000000	75,678969	58,700125	35,093333		
9	9,000000	75,678969	58,700125	35,093333		
10	10,000000	75,678969	58,700125	35,093333		
11	11,000000	75,678969	58,700125	35,093333		
12	12,000000	75,678969	58,700125	35,093333		

- Extract the first 5 columns, they show the slice position (T), the X and Y coordinates and the inferred Z position (Z average).

A	B	C	D	E		
1	index	T	X (μm)	Y (μm)	Z average	fact
2	0	0	75.67897	58.70013	35.09333	35.
3	1	1	75.67897	58.70013	34.645	34.
4	2	2	75.67897	58.70013	35.09333	35.
5	3	3	75.67897	58.70013	35.09333	35.
6	4	4	75.67897	58.70013	35.09333	35.
7	5	5	75.67897	58.70013	35.3175	35.
8	6	6	75.67897	58.70013	35.09333	35.
9	7	7	75.67897	58.70013	35.09333	35.
10	8	8	75.67897	58.70013	35.09333	35.
11	9	9	75.67897	58.70013	35.09333	35.
12	10	10	75.67897	58.70013	35.09333	35.
13	11	11	75.67897	58.70013	35.09333	35.
14	12	12	75.67897	58.70013	35.09333	35.
15	13	13	75.67897	58.70013	35.09333	35.
16	14	14	75.67897	58.70013	35.09333	35.
17	15	15	75.67897	58.70013	35.09333	35.
18	16	16	75.67897	58.70013	35.09333	35.
19	17	17	75.67897	58.70013	34.645	34.
20	18	18	75.67897	58.70013	35.09333	35.

- If you wish to programmatically retrieve the bead's x,y,z-position from the outputfile with ending "...\_s" of MultiFocalParticleTracker-Complex-3 you may have a look on the MATLAB script plotting bead tracks (<https://github.com/hansenjn/MultifocalImaging-AnalysisToolbox/tree/master/Matlab%20scripts>).
- The inferred z positions in relationship to the piezo position (indicated by the values in column "T" if multiplied by the step-size of the piezo during recording) should reveal a linear relationship with a slope of unity. The standard deviation of the residuals from a linear fit with slope unity demonstrates how accurate the method is. The average of the residuals should be zero to confirm that the method reveals an unbiased inferred z-position.
  - Example analysis of an exemplary bead:



### 3D Bead tracking

When the setup has been calibrated by generating and verifying a LUT file (see previous two chapters), bead analysis can be conducted in multifocal time-lapse images acquired with exactly the same setup as the setup used to determine the LUT file. Preprocess the image stack as described in the beginning of the chapter "Determine a calibration Look-Up-Table (LUT)": prepare the image (as described in the chapter "Preparing the data for calibration and analysis").

Next, generate a text file containing the bead positions over time using TrackMate (<https://imagej.net/TrackMate>) as explained in the following. It is recommended to not install TrackMate

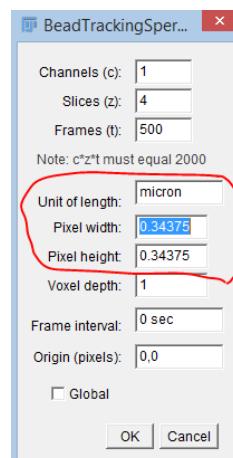
More information, license notes, credits: <https://github.com/hansenjn/MultifocalImaging-AnalysisToolbox>

manually into your ImageJ but rather download and use the FIJI distribution of ImageJ, where the plugin TrackMate is included, because TrackMate requires the installation of additional libraries, making manual installation more complicated (<https://imagej.net/Fiji/Downloads>).

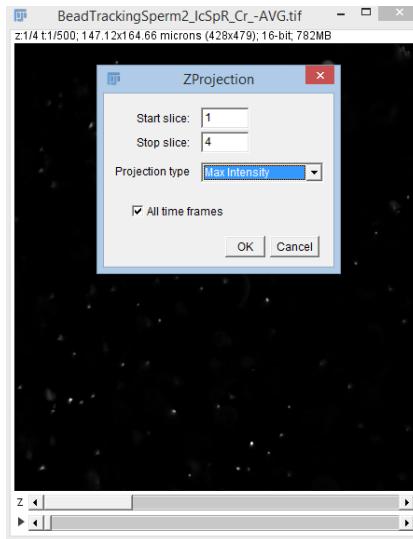
- Launch FIJI and open the prepared image series you aim to analyze



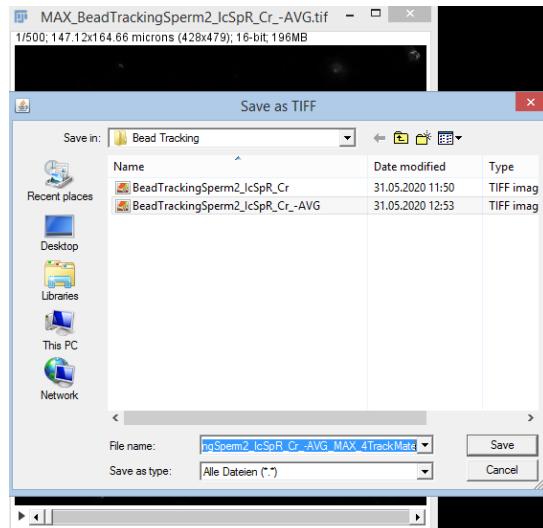
- Make sure the image is correctly calibrated: Image > Properties; If the information provided at “Unit of length”, “Pixel width”, and “Pixel height” is incorrect, correct it; Press OK.



- Create a Maximum Projection of the image: Image > Stacks > Z Project ...



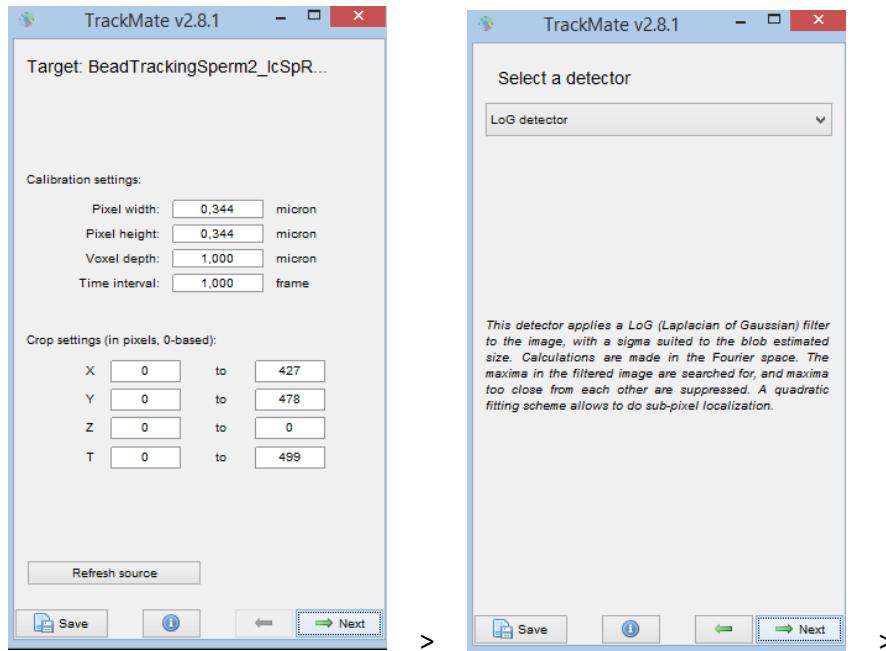
- Save the stack with the ending “\_MAX\_4TrackMate.tif” to your directory: File > Save As > Tiff...



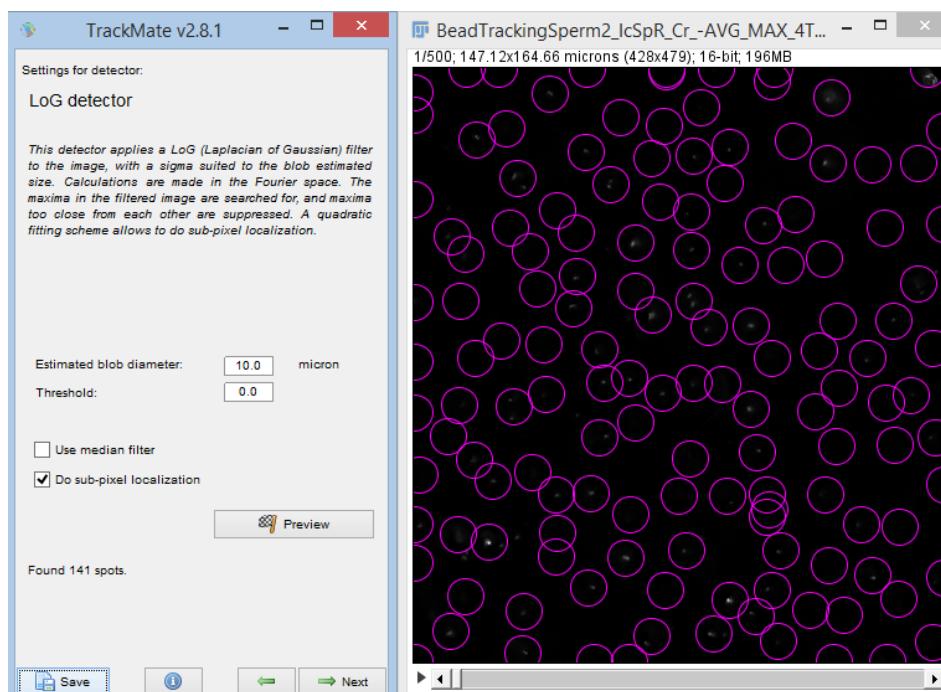
- Launch TrackMate: Plugins > Tracking > TrackMate; if a dialog pops up asking you to swap Z and T confirm by pressing Yes (or “Ja” if you have a German computer):



- A dialog pops up - press Next:

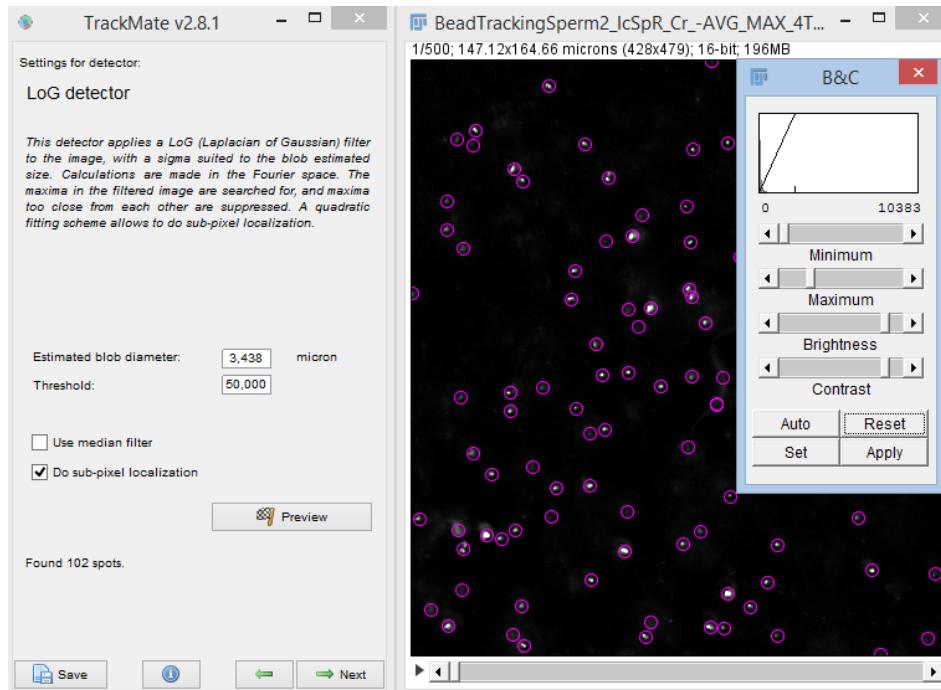


- Press Next and then “Preview” to see the positions that will be detected as a particle (will be encircled in purple)

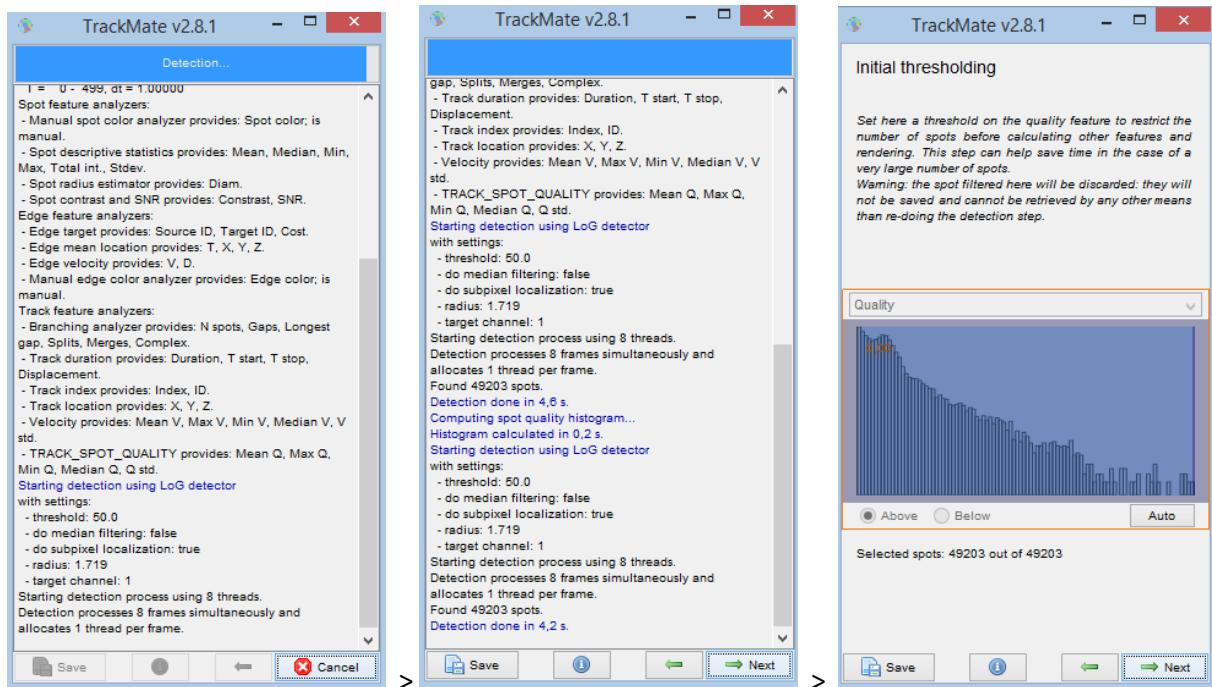


- Adapt the blob diameter and the threshold until the detection is correct (press preview to preview whether settings have improved detection). Eventually, you might need to increase the Brightness / Contrast (Image > Adjust > Brightness / Contrast) to better see where beads are located.

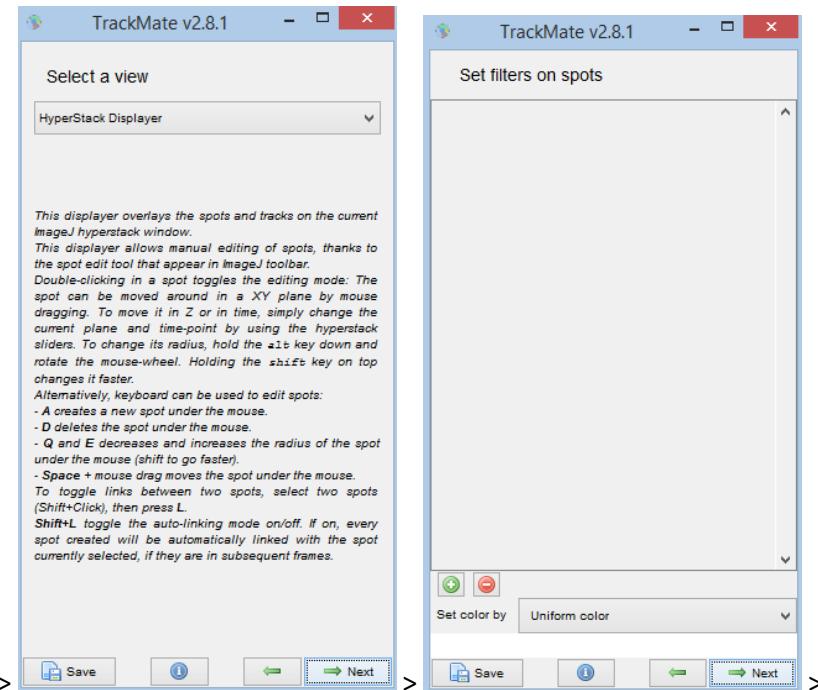
More information, license notes, credits: <https://github.com/hansenjn/MultifocalImaging-AnalysisToolbox>



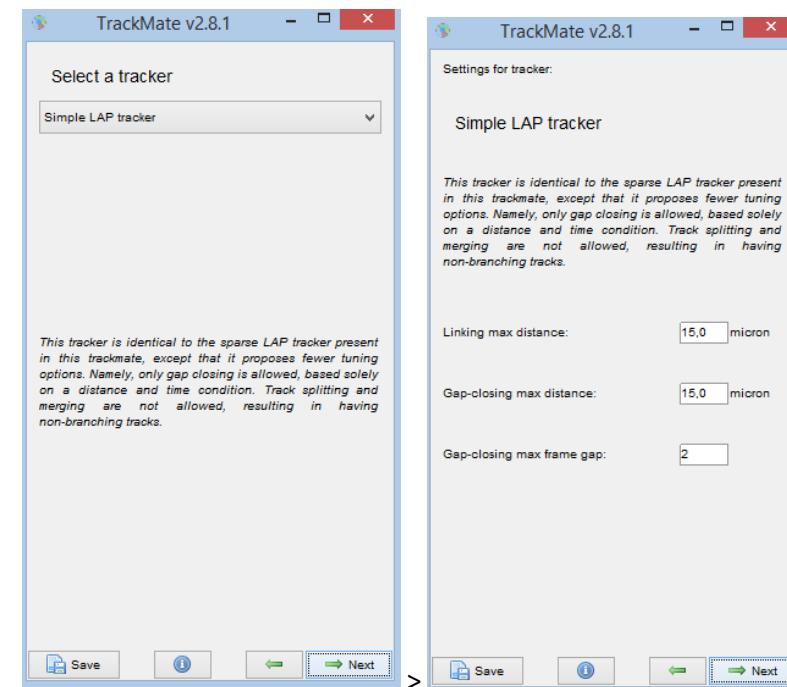
- Click five times Next until you reach a dialog showing “Select Tracker”:



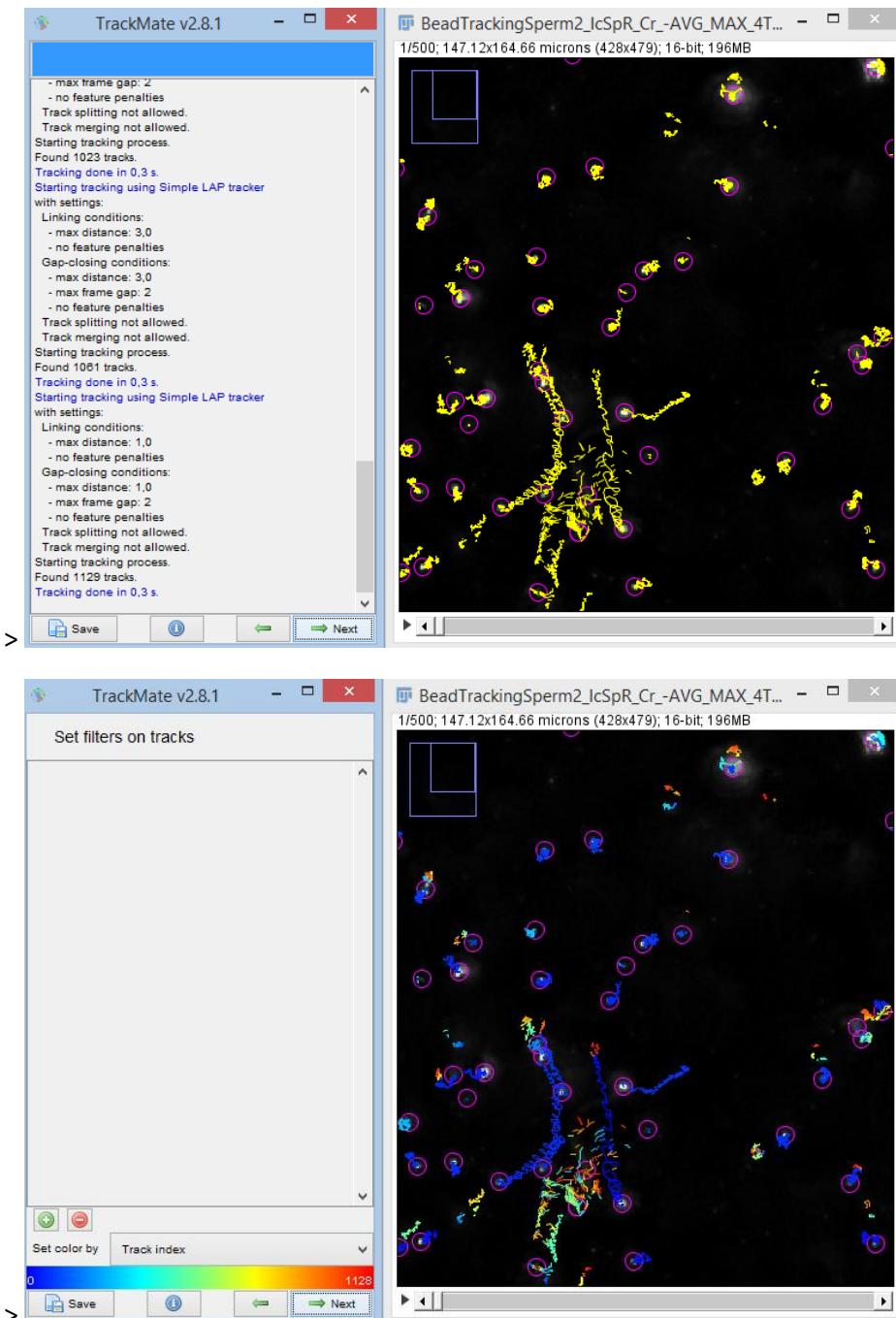
More information, license notes, credits: <https://github.com/hansenjn/MultifocalImaging-AnalysisToolbox>



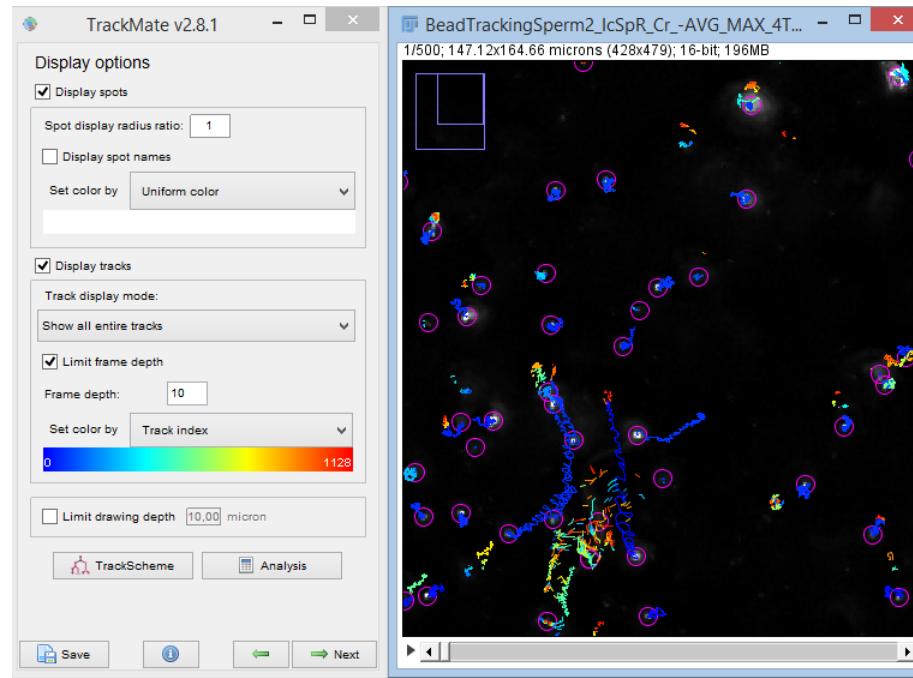
- In the dialog “Select a tracker”, select “Nearest neighbor search” and press Next for four times:



More information, license notes, credits: <https://github.com/hansenjn/MultifocalImaging-AnalysisToolbox>



More information, license notes, credits: <https://github.com/hansenjn/MultifocalImaging-AnalysisToolbox>



- Note: You may adapt all the filtering settings, the tracking settings, etc. in TrackMate to improve your tracking. TrackMate offers a variety of tools to filter out false-detected particles, short tracks, etc. (<https://imagej.net/TrackMate>)
- Press "Analysis" and three windows pop up:

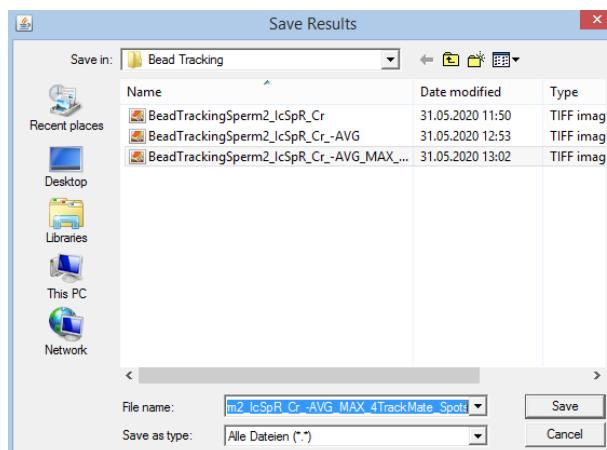
Three tables are shown side-by-side, each with a 'File' menu at the top. The first table, 'Track statistics', has columns: Label, NUMBER\_SPOTS, NUMBER\_GAPS, LONG. The second, 'Links in tracks statistics', has columns: Label, TRACK\_ID, SPOT\_SOURCE\_ID. The third, 'Spots in tracks statistics', has columns: Label, ID, TRACK\_ID, QUALITY, POSITION. All three tables have 10 rows of data.

Label	NUMBER_SPOTS	NUMBER_GAPS	LONG
1 Track_2897	326	0	0
2 Track_2898	376	1	1
3 Track_2899	500	0	0
4 Track_2900	500	0	0
5 Track_2901	12	0	0
6 Track_2902	500	0	0
7 Track_2903	500	0	0
8 Track_2904	500	0	0
9 Track_2905	3	0	0
10 Track_2906	366	0	0

Label	TRACK_ID	SPOT_SOURCE_ID
1 (ID49827 : ID49924)	0	49827
2 (ID49924 : ID49629)	0	49924
3 (ID49629 : ID50020)	0	49629
4 (ID50020 : ID50116)	0	50020
5 (ID50116 : ID49726)	0	50116
6 (ID49726 : ID49533)	0	49726
7 (ID49533 : ID50215)	0	49533
8 (ID50215 : ID50311)	0	50215
9 (ID50311 : ID50602)	0	50311
10 (ID50602 : ID50475)	0	50602

Label	ID	TRACK_ID	QUALITY	POSITION
1 ID49827	49827	0	1053.79675	87.24324
2 ID49924	49924	0	1007.56110	87.29112
3 ID49629	49629	0	1136.19006	87.31785
4 ID50020	50020	0	999.36249	87.28498
5 ID50116	50116	0	1340.79919	87.33327
6 ID49726	49726	0	1342.32104	87.33234
7 ID49533	49533	0	1336.68665	87.31784
8 ID50215	50215	0	1403.55603	87.35430
9 ID50311	50311	0	1300.04761	87.34724
10 ID50602	50602	0	1350.52222	87.43795

- Close the windows "Track statistics" and "Links in tracks statistics" without saving, select the window "Spots in tracks statistics" and save it as a .csv file: File > Save As ...

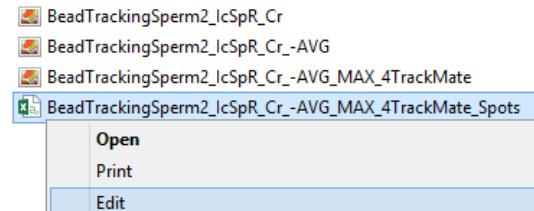


- Close all images and windows in Fiji.

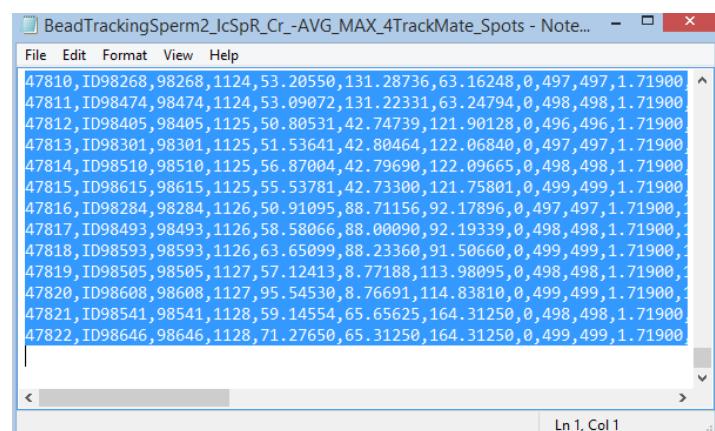
More information, license notes, credits: <https://github.com/hansenjn/MultifocalImaging-AnalysisToolbox>

- Extract the points from the .csv file and save them into a text file (one row per position, in each row: <x-position> tab <y-position>). This can be done manually using a table-calculation-software like Excel or programmatically in MatLab or R by importing the .csv file, extracting the X and Y positions ("Position\_X", "Position\_Y" in the file) for the timepoint ("Position\_T") 0, and saving them automatically into a text file. See here an exemplary way to extract the positions in Windows and using Excel:

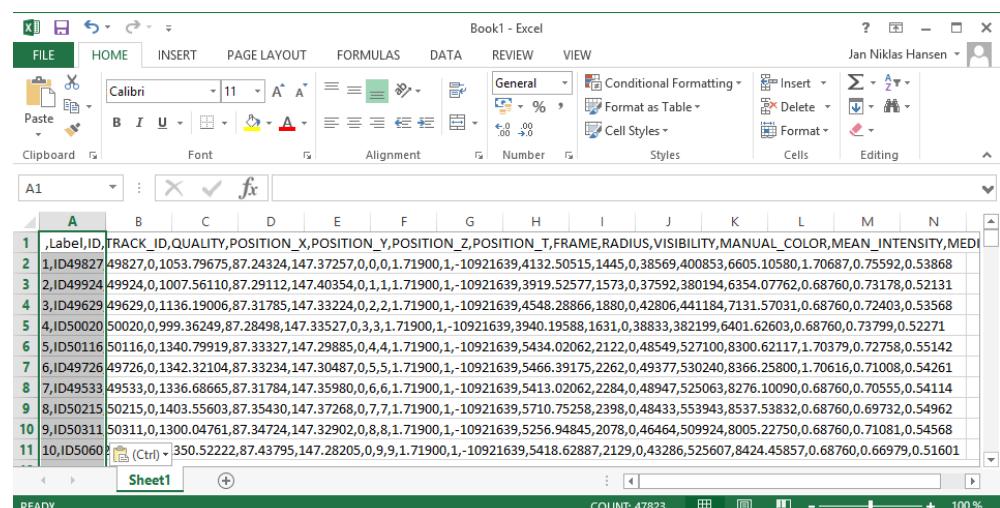
- Open the saved .csv file with a text editor (right-click on file > Edit)



- Mark all and press Ctrl + C:



- Open Excel or a similar table calculation software and press Ctrl + V

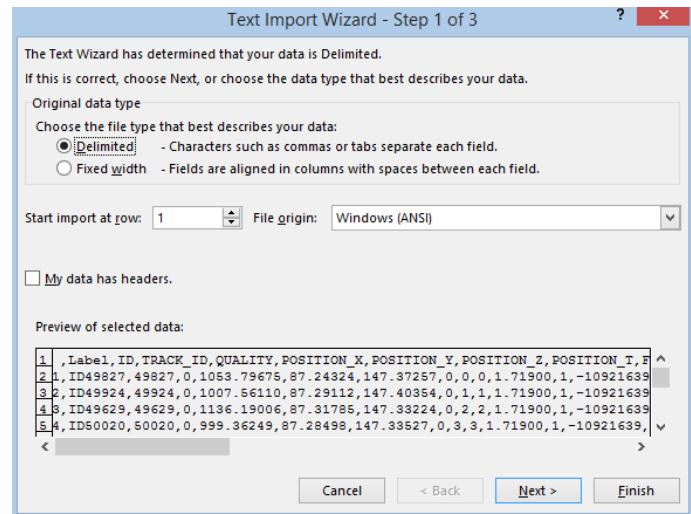


- Click on the small Ctrl button on the bottom and select "Use Text Import Wizard..."

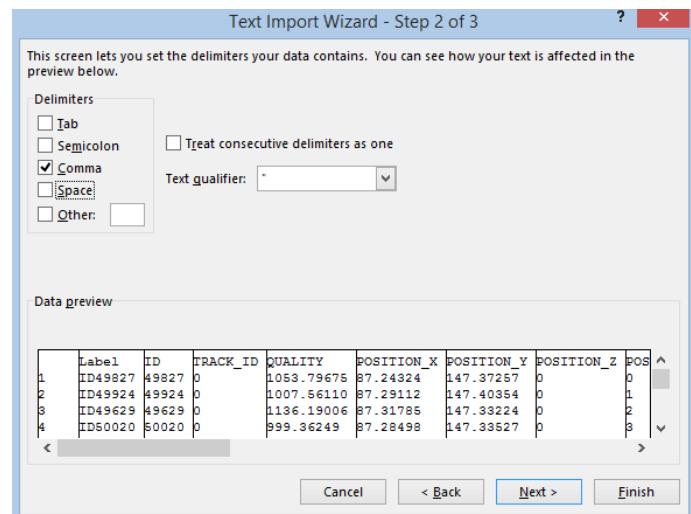
More information, license notes, credits: <https://github.com/hansenjn/MultifocalImaging-AnalysisToolbox>

10	9, ID50311	50311, 0, 1300.04761, 87.34724, 14
11	10, ID5060	350.52222, 87.43795, 1

- Select Delimited and press Next



- Select Comma (see below) and press Finish



- Copy the columns POSITION\_T, POSITION\_X, POSITION\_Y into a new table (order T, X, Y).

More information, license notes, credits: <https://github.com/hansenjn/MultifocalImaging-AnalysisToolbox>

The screenshot shows two Microsoft Excel spreadsheets side-by-side. The left spreadsheet, 'Sheet1', has a header row and 17 data rows. The right spreadsheet, 'Sheet2', has a header row and 11 data rows. Both spreadsheets show the same columns: POSITION\_T, POSITION\_X, and POSITION\_Y.

	A	B	C	D	E	F	G	H	I	J	
1	Label	ID	TRACK_ID	QUALITY	POSITION_X	POSITION_Y	POSITION_T	POSITION_I	FRAME	PAI	
2	1	ID49827	49827	0	1053.8	87.24324	147.37257	0	0	0	
3	2	ID49324	49324	0	1007.6	87.29112	147.40354	0	1	1	
4	3	ID49629	49629	0	1136.2	87.31785	147.33224	0	2	2	
5	4	ID50020	50020	0	993.36	87.28498	147.33527	0	3	3	
6	5	ID50116	50116	0	1340.8	87.33327	147.29885	0	4	4	
7	6	ID49726	49726	0	1342.3	87.33234	147.30487	0	5	5	
8	7	ID49533	49533	0	1336.7	87.31784	147.35938	0	6	6	
9	8	ID50215	50215	0	1403.6	87.3543	147.37268	0	7	7	
10	9	ID50311	50311	0	1300	87.34724	147.32902	0	8	8	
11	10	ID50602	50602	0	1350.5	87.43795	147.28205	0	9	9	
12	11	ID50475	50475	0	1339.1	87.3945	147.28699	0	10	10	
13	12	ID50505	50505	0	1540	87.47731	147.31607	0	11	11	
14	13	ID50702	50702	0	1474.3	87.53394	147.35636	0	12	12	
15	14	ID50800	50800	0	1542.3	87.54117	147.24707	0	13	13	
16	15	ID50836	50836	0	1514.7	87.47944	147.19875	0	14	14	
17	16	ID50947	50947	0	1514.8	87.47649	147.19489	0	15	15	

	A	B	C	D	E
1	POSITION_T	POSITION_X	POSITION_Y		
2	0	87.24324	147.37257		
3	1	87.29112	147.40354		
4	2	87.31785	147.33224		
5	3	87.28498	147.33527		
6	4	87.33327	147.29885		
7	5	87.33234	147.30487		
8	6	87.31784	147.35938		
9	7	87.3543	147.37268		
10	8	87.34724	147.32902		
11	9	87.43795	147.28205		
12	10	87.3945	147.28699		
13	11	87.47731	147.31607		
14	12	87.53394	147.35636		
15	13	87.54117	147.24707		
16	14	87.47944	147.19875		
17	15	87.47649	147.19489		
18	16	87.47137	147.24988		
19	17	87.48654	147.31544		
20	18	87.49124	147.32594		
21	19	87.47473	147.30214		
22	20	87.51831	147.29336		
23	21	87.54533	147.3368		

- Copy the newly generated table without the head row (row 1) into a new text file and save the text file as “<image name>\_4TrackMate\_Spots.txt”.

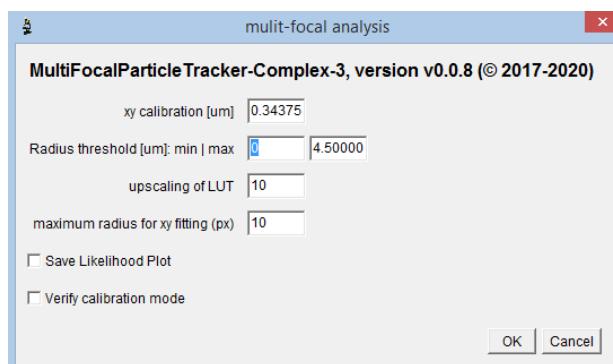
The screenshot shows Microsoft Excel with two sheets and a Notepad window. The Notepad window displays a list of tracks with their X and Y coordinates.

	A	B	C	D	E
1	POSITION_T	POSITION_X	POSITION_Y		
2	0	87.24324	147.37257		
3	1	87.29112	147.40354		
4	2	87.31785	147.33224		
5	3	87.28498	147.33527		
6	4	87.33327	147.29885		
7	5	87.33234	147.30487		
8	6	87.31784	147.35938		
9	7	87.3543	147.37268		
10	8	87.34724	147.32902		
11	9	87.43795	147.28205		
12	10	87.3945	147.28699		
13	11	87.47731	147.31607		
14	12	87.53394	147.35636		
15	13	87.54117	147.24707		
16	14	87.47944	147.19875		
17	15	87.47649	147.19489		
18	16	87.47137	147.24988		
19	17	87.48654	147.31544		
20	18	87.49124	147.32594		
21	19	87.47473	147.30214		
22	20	87.51831	147.29336		
23	21	87.54533	147.3368		

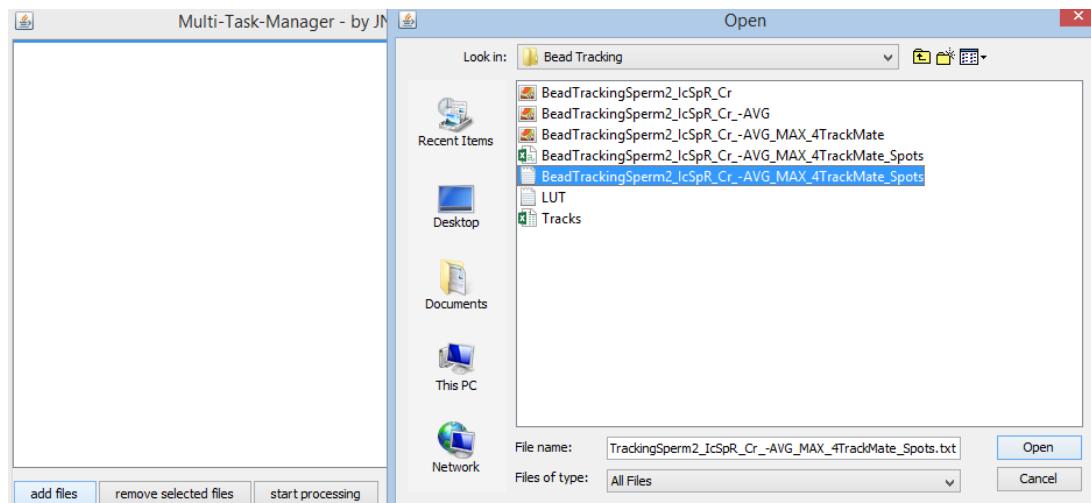
Now that the text file containing the bead positions over time has been generated, analysis of the Z position can be performed.

- Open ImageJ
- If not already installed (this should have been installed for verifying the LUT), install the latest release of the plugin MultiFocalParticleTracker-Complex-3 (<https://github.com/hansenjn/MultiFocalParticleTracker-Complex-3/releases/>) and restart ImageJ
- Launch MultiFocalParticleTracker-Complex-3: Plugins > JNH > Multi Focal > Complex Particle Tracking 3...
- Enter the settings according to your analysis (enter the same settings as you used during production and verification of the LUT!)
  - Xy calibration [μm]: enter the xy calibration of your image – for the exemplary data set: 0.34375 μm / px

- Radius threshold [um] ...: allows to exclude determined width values below the min and above the max threshold from including them into calculating the z-position. If no threshold shall be used – as in the exemplary analysis – set the min to 0 and the max to a value above the highest value in your LUT.
- Upscaling of LUT: To obtain a more precise z estimation, a spline interpolation can be used to add intermediate steps between the z-position-width-values indicated in the LUT. E.g. when setting the upscaling value to 10, between two lines in the LUT, 9 more lines are added with equidistant steps. The width values for these extra steps are determined by spline interpolation from the neighbored widths values.
- Maximum radius for xy fitting: Enter the radius that you have entered in MultiFocalParticleTracker-Calibration-3 during LUT generation: for the exemplary data set: 10 px
- Save Likelihood Plot: do not check / ignore
- Verify calibration mode: do not check

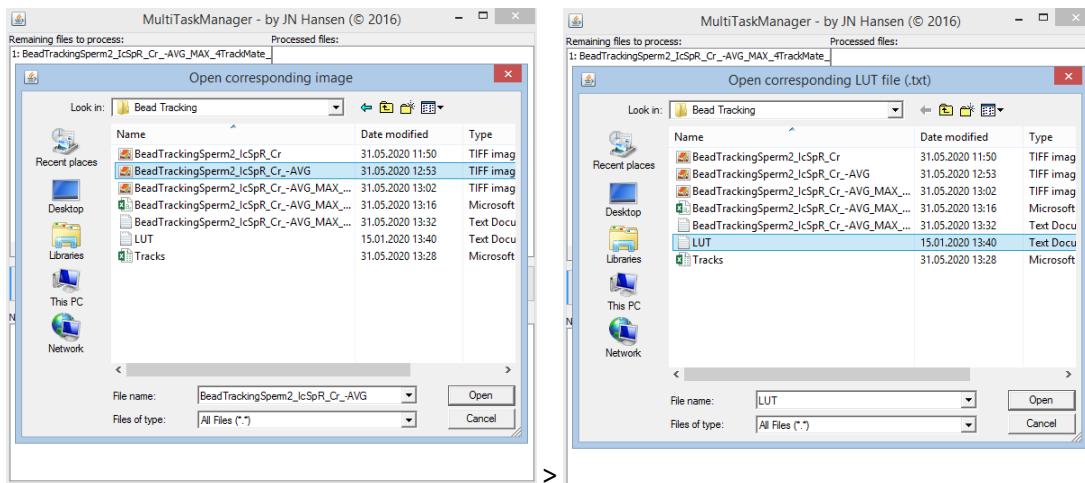


- A dialog pops up: add the text file containing the bead positions and press “start processing”:

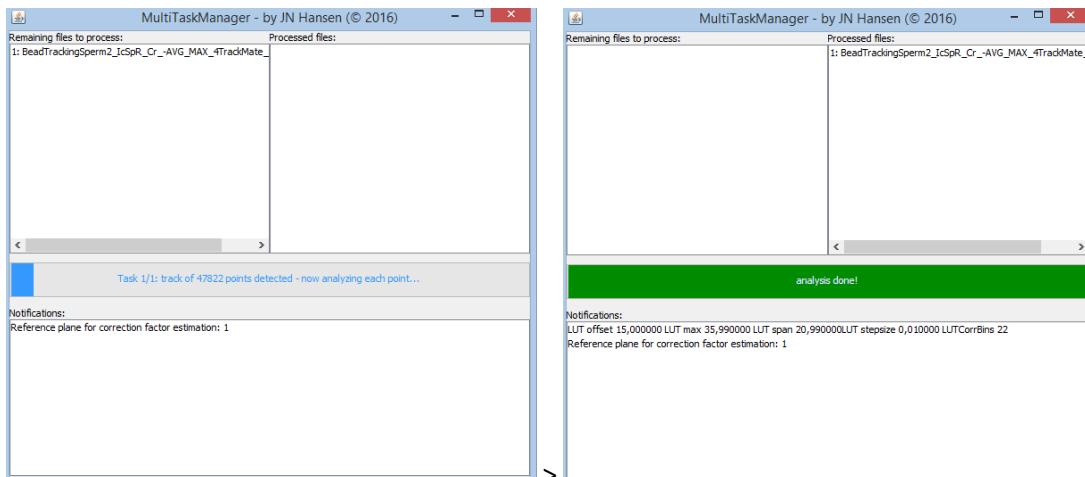


- A dialog pops up requesting to open the corresponding image. Select the corresponding image and press Open. Next, dialog pops up requesting you to load the LUT. Select the LUT and press Open.

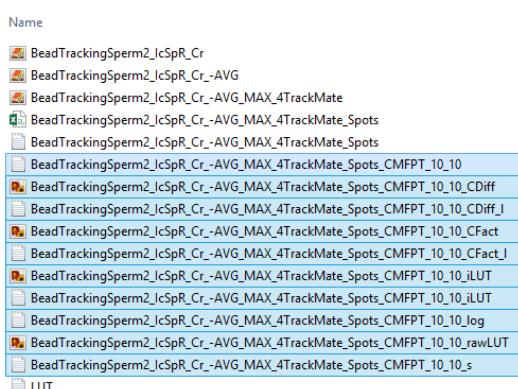
More information, license notes, credits: <https://github.com/hansenjn/MultifocalImaging-AnalysisToolbox>



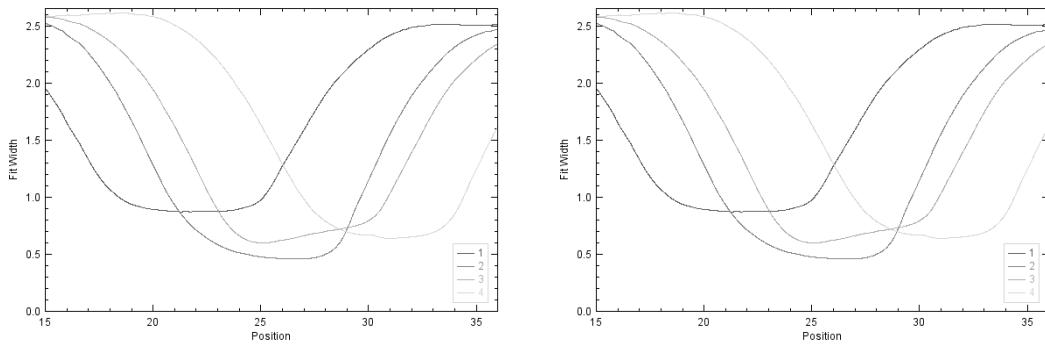
- Wait until MultiTaskManager states “analysis done!”



- The plugin has saved new files to the folder where the text file with bead position was saved. They all contain an additional suffix (“\_CMFPT\_<numbers>\_”) and specific file endings.



- ...rawLUT.png shows a plot of the LUT before and ...iLUT.png after spline interpolation:



- Open the file with ending "...\_s.txt" and copy the content e.g. to excel to investigate the results.

BeadTrackingSperm2_IcSpR_Cr_AVG_MAX_4TrackMate_Spots_CMFPT_10_10_s					
BeadTrackingSperm2_IcSpR_Cr_AVG_MAX_4TrackMate_Spots_CMFPT_1...					
File	Edit	Format	View	Help	
index	T	X ( $\mu\text{m}$ )	Y ( $\mu\text{m}$ )	Z average ( $\mu\text{m}$ )	factor-corr. Z avg ( $\mu\text{m}$ )
0	0,000000	87,243240	147,372570	24,330000	
1	1,000000	87,291120	147,403540	24,405000	
2	2,000000	87,317850	147,332240	24,395000	
3	3,000000	87,284980	147,335270	24,345000	
4	4,000000	87,333270	147,298850	24,457500	
5	5,000000	87,332340	147,304870	24,537500	
6	6,000000	87,317840	147,359800	24,432500	
7	7,000000	87,354300	147,372680	24,447500	
8	8,000000	87,347240	147,329020	24,395000	
9	9,000000	87,437950	147,282050	24,245000	
10	10,000000	87,394500	147,286990	24,312500	
11	11,000000	87,477310	147,316070	24,612500	
12	12,000000	87,533940	147,356360	24,700000	

- Extract the first 5 columns, they show a unique measurement ID for each measurement (index), the slice position (T), the X and Y coordinates and the inferred Z position (Z average).

A	B		C	D	E	
1	index	T	X ( $\mu\text{m}$ )	Y ( $\mu\text{m}$ )	Z average	fac
2	0	0	87.243	147.373	24.330	
3	1	1	87.291	147.404	24.405	
4	2	2	87.318	147.332	24.395	
5	3	3	87.285	147.335	24.345	
6	4	4	87.333	147.299	24.458	
7	5	5	87.332	147.305	24.538	
8	6	6	87.318	147.360	24.433	
9	7	7	87.354	147.373	24.448	
10	8	8	87.347	147.329	24.395	

- If you wish to programmatically retrieve the bead's t,x,y,z-position from the outputfile with ending "...\_s" of MultiFocalParticleTracker-Complex-3, have a look on the MATLAB script plotting bead tracks (<https://github.com/hansenjn/MultifocalImaging-AnalysisToolbox/tree/master/Matlab%20scripts>). The MATLAB script additionally implements connecting the bead positions to tracks, filtering the tracks, and plotting the tracks.

## 3D Flagellar Reconstruction

### Requirements

USER GUIDE WRITING TO BE CONTINUED