

Multifocal Image Analysis – User Guide

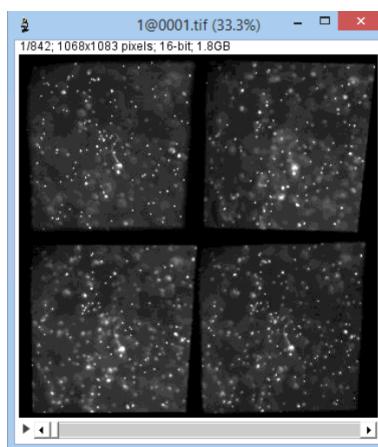
Introduction

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>.

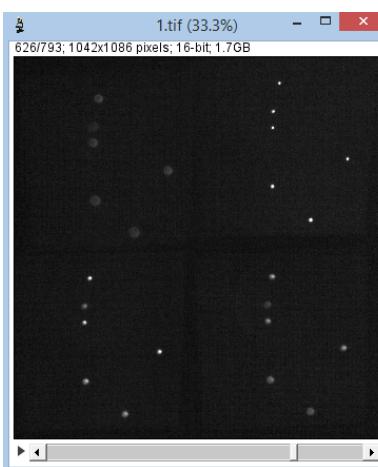
3D tracking of beads

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



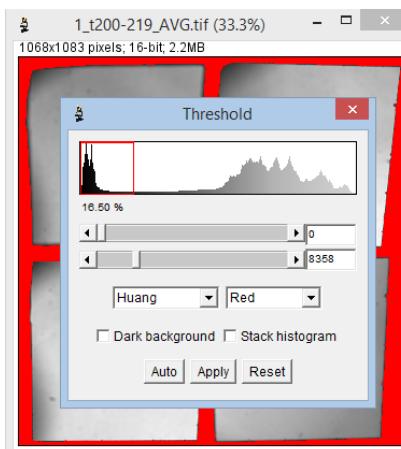
3. A reference file for intensity corrections (obtained by imaging without a specimen)



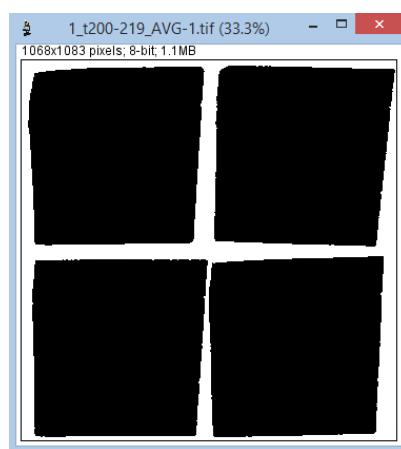
Workflow

Creating an intensity 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

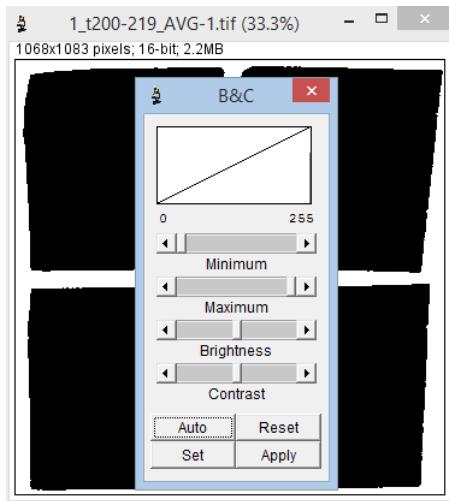


- The duplicate image will look like this:

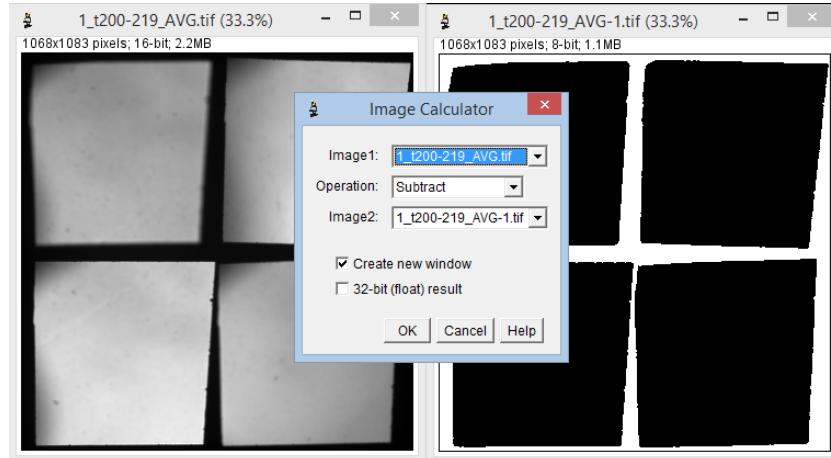


- Convert the image to 16-bit: Image > Type > 16-bit
- Reset the intensity values: Image > Adjust > Brightness & Contrast, Press Apply

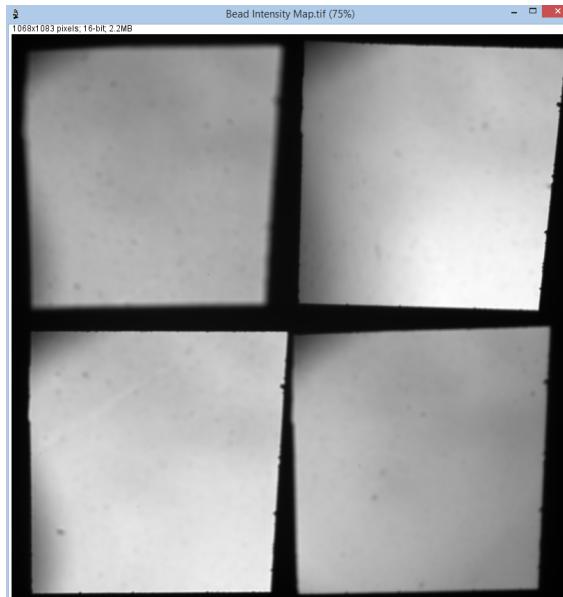
More information, license notes, credits: <https://github.com/hansenjn/MultifocalImaging-AnalysisToolbox>



- 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

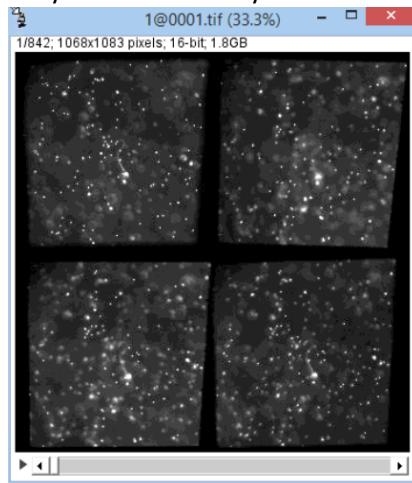


- Save the generated image as “Bead Intensity Map.tif”, it will serve as a map for intensity corrections in the plugin “Multifocal Preparation”.

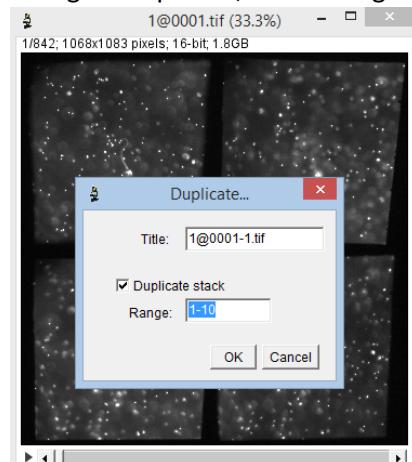


Creating a registration file

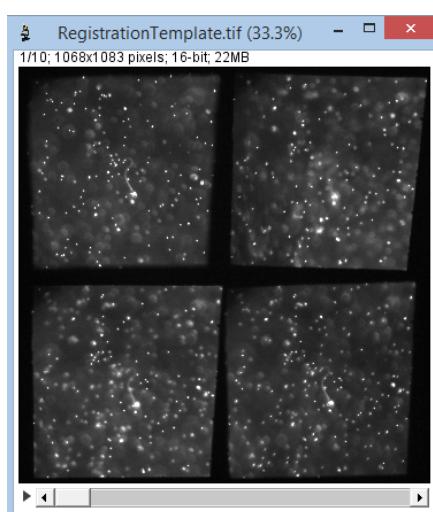
- Install the plugin MultiStackReg from Brad Busse: <http://bradbusse.net/downloads.html>
- Install the latest release from the plugin Multifocal_Preparation: https://github.com/hansenjn/MultiFocal_Preparation/releases
- Restart ImageJ
- Open the multifocal time series you want to analyze



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

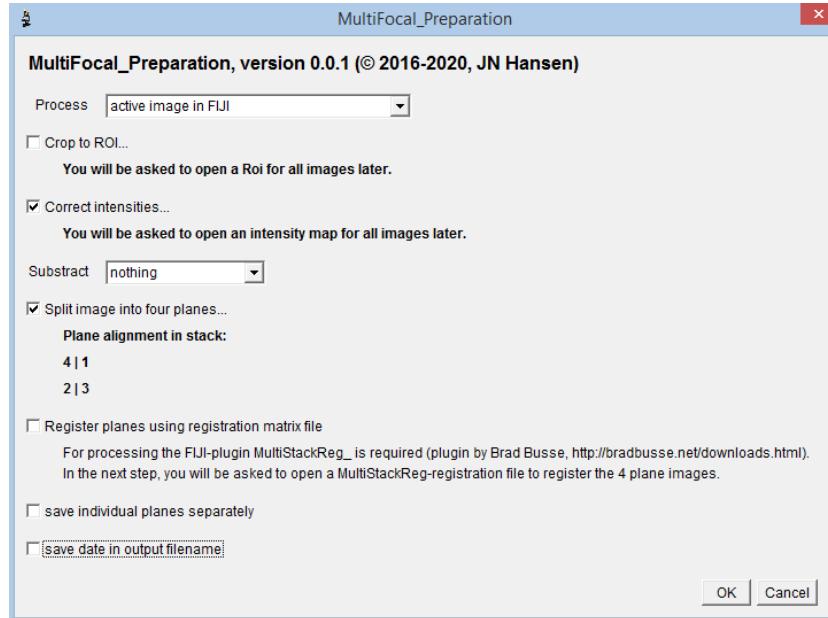


- Save the image as "RegistrationTemplate.tif"

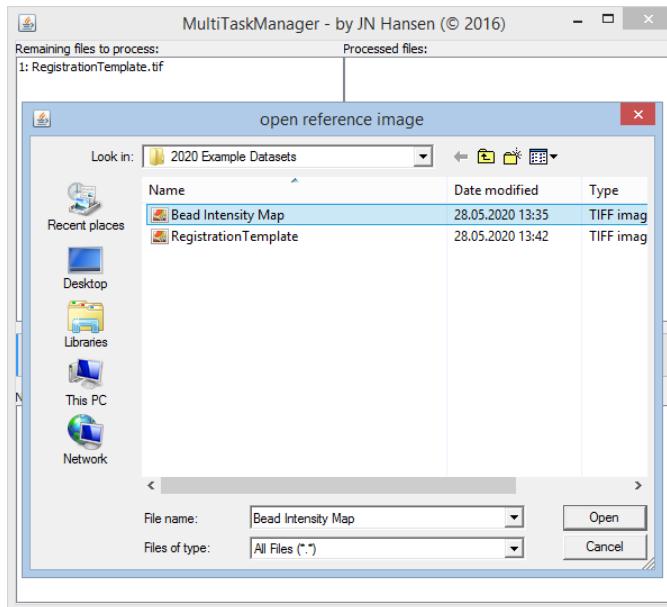


More information, license notes, credits: <https://github.com/hansenjn/MultifocalImaging-AnalysisToolbox>

- 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:

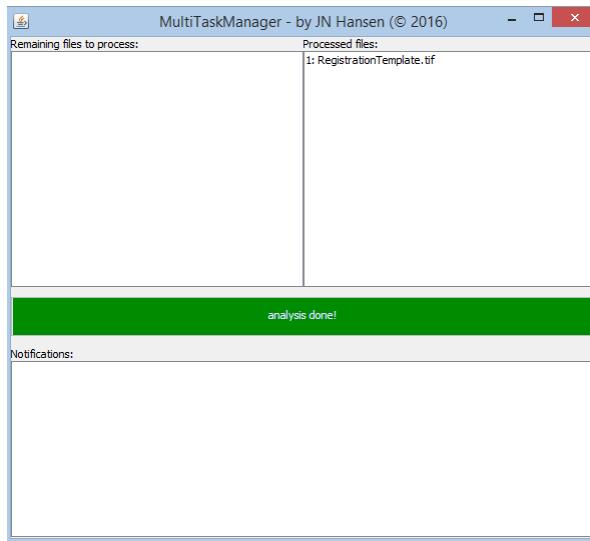


- Wait until the Processing is Done
 - In brief, the plugin will correct the intensities according to the reference image and then split the image into 4 equal quarters
 - Each quarter becomes a separate plane in the output Hyperstack
 - The conversion is as follows: the upper right quarter becomes plane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

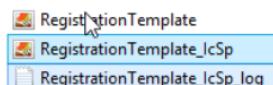
Plane alignment in stack:
4|1
2|3

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

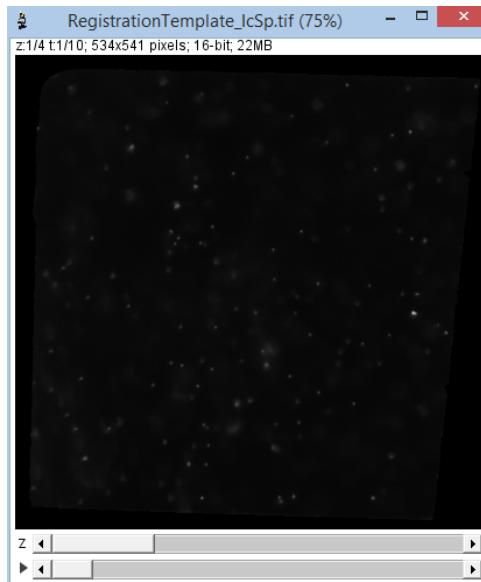
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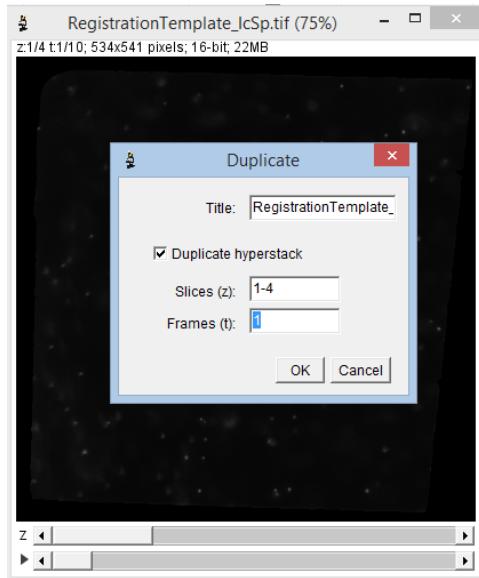
- Then, new files have been saved to the repository where the RegistrationTemplate.tif file was saved.



- 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



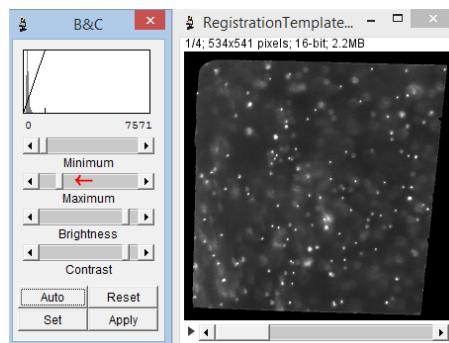
- You can see that each focal plane has become a different Z slice.
- Extract a single timepoint: Image > Duplicate, select the following settings, press OK



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

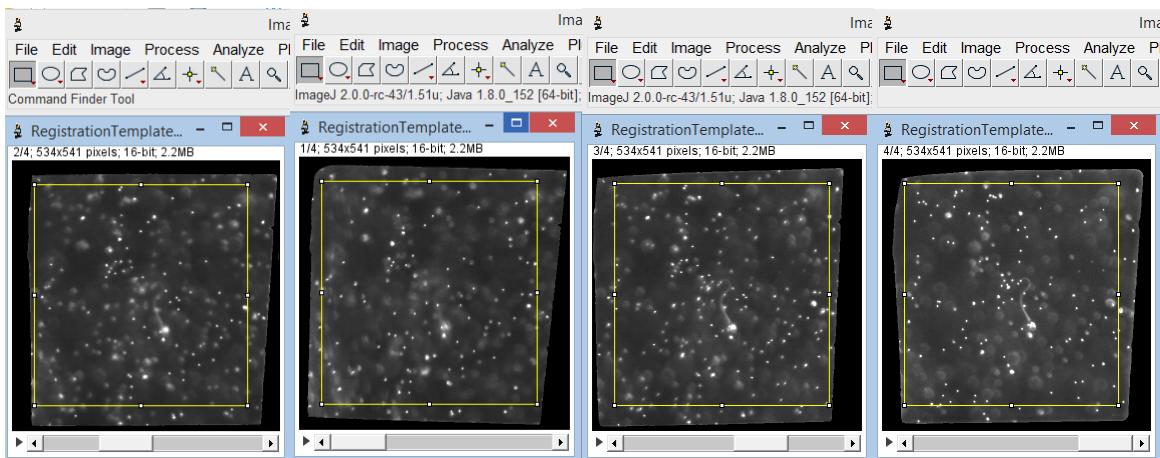


- Adapt the display range so that you can see the borders of the planes: Image > Adjust > Brightness/Contrast, drag down the maximum so that edges become apparent in the image

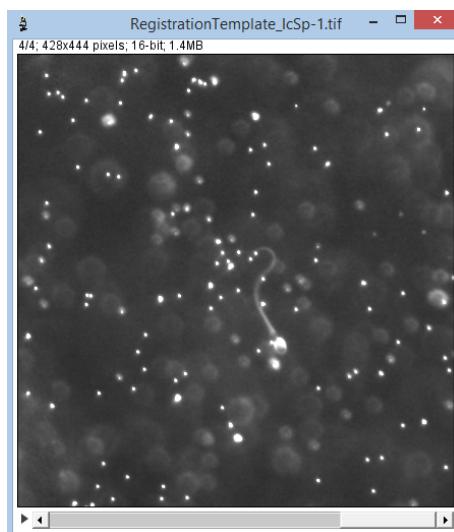


- Select the Rectangle tool in the ImageJ bar and draw a selection that is inside all planes and does not contain any black areas. Check the different planes by scrolling with the mouse wheel and adapting the selection.

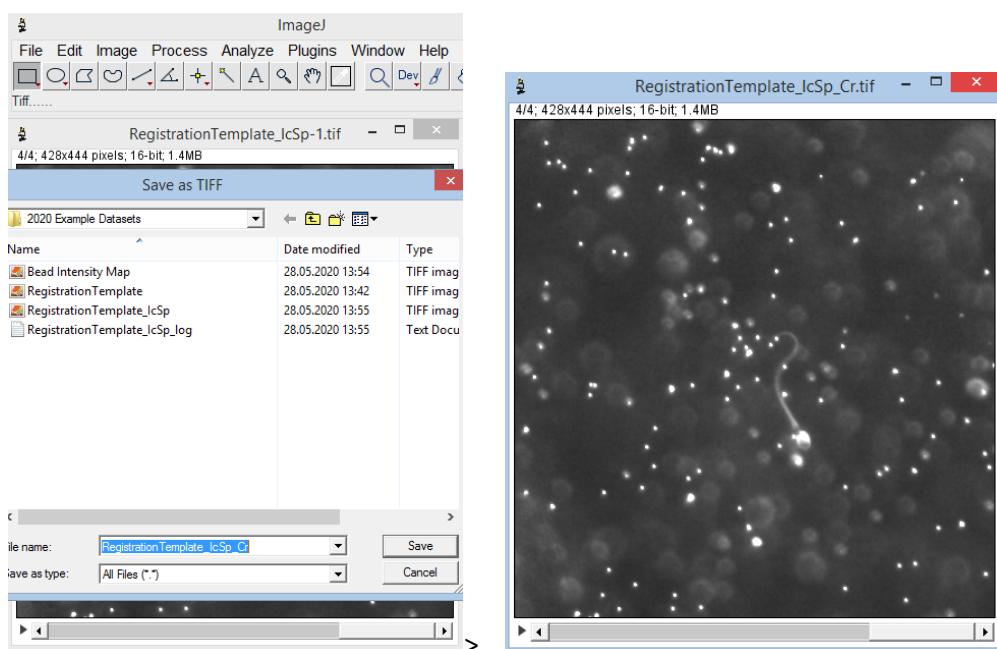
More information, license notes, credits: <https://github.com/hansenjn/MultifocalImaging-AnalysisToolbox>



- Crop the stack to the selection: Image > Crop

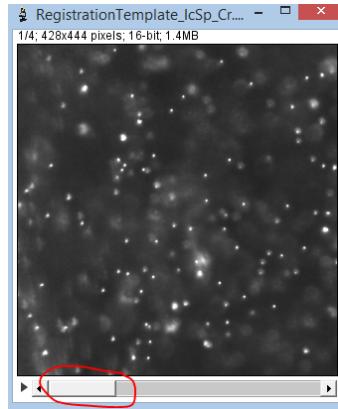


- Save the stack as RegistrationTemplate_IcSp_Cr: File > Save As ... > Tiff

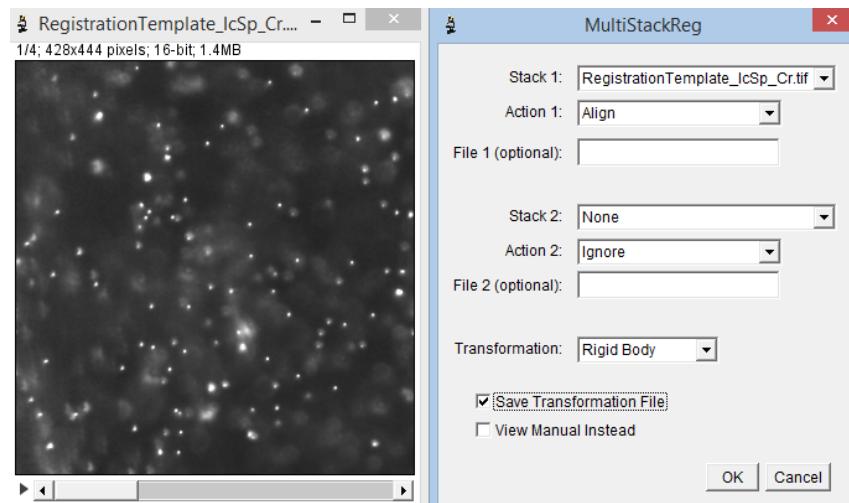


More information, license notes, credits: <https://github.com/hansenjn/MultifocalImaging-AnalysisToolbox>

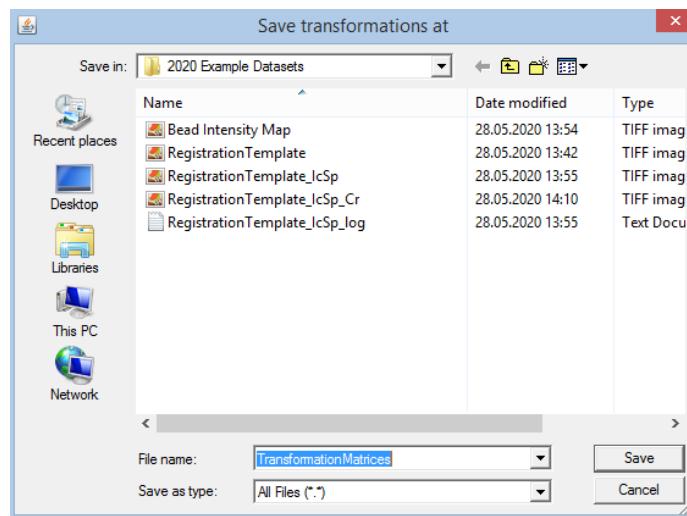
- Make sure that the first plane is selected



- 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

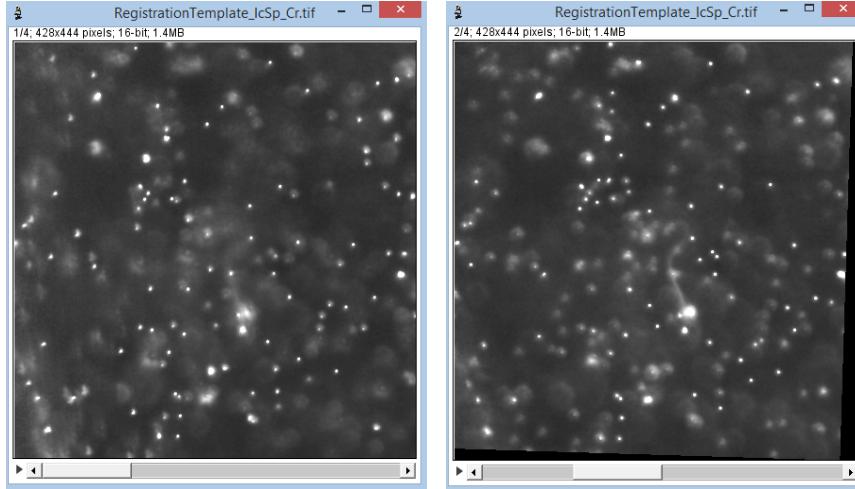


- Wait until the plugin is done and the status bar looks “resting”/normal again

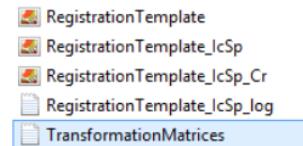


More information, license notes, credits: <https://github.com/hansenjn/MultifocalImaging-AnalysisToolbox>

- 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:



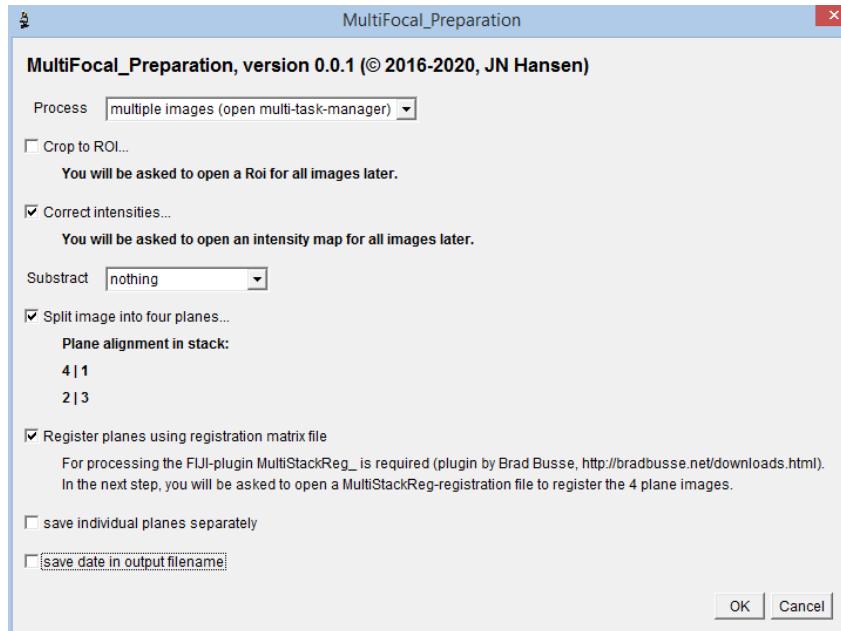
- The matrix file will be needed as a reference for registering the planes using the MultiFocal_Preparation plugin.



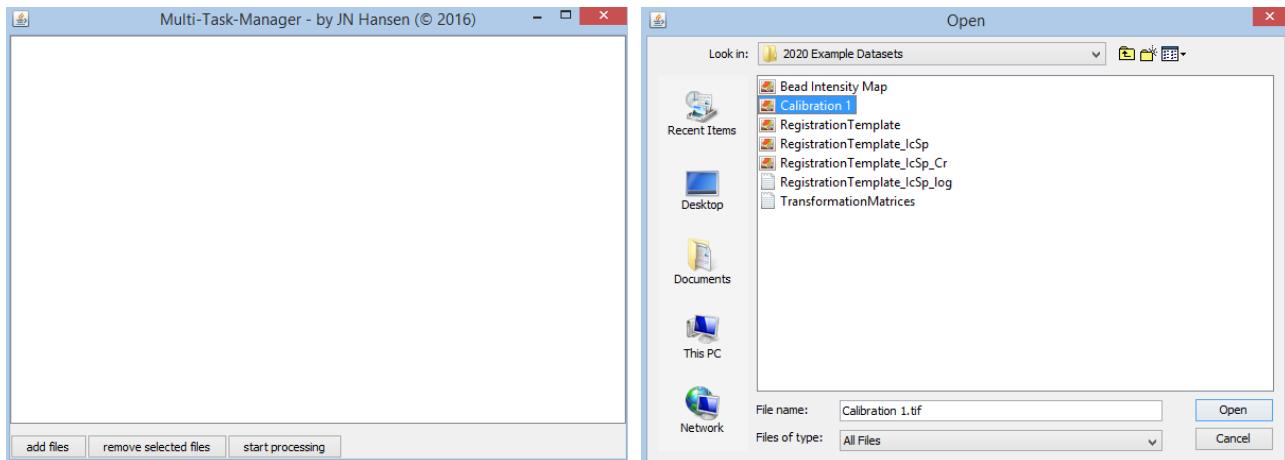
More information, license notes, credits: <https://github.com/hansenjn/MultifocalImaging-AnalysisToolbox>

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.

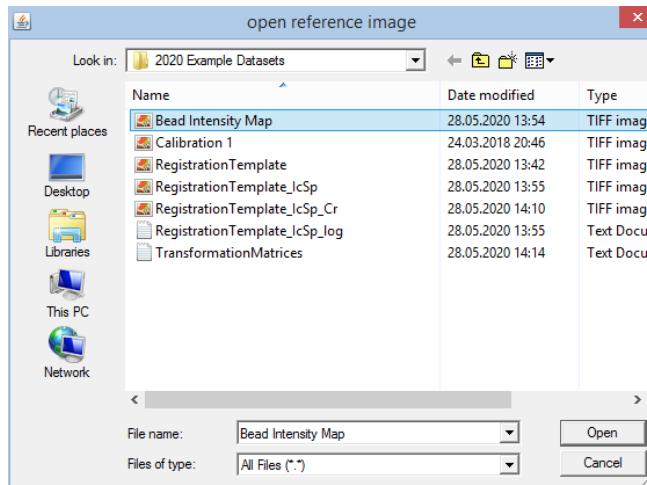


- A dialog pops up, press add files, select the files you want to analyze in your file system, press open (eventually repeat to add more files from different repositories), press start processing to let MultiFocal_Preparation prepare the data.

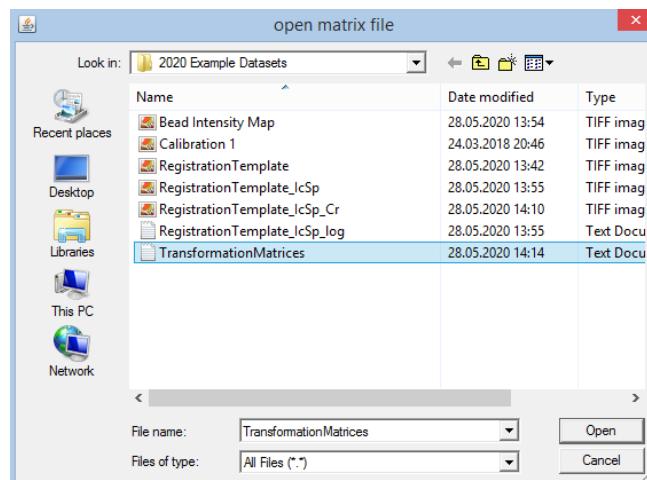


- A dialog pops up, select the Bead Intensity Map as a reference image and press open:

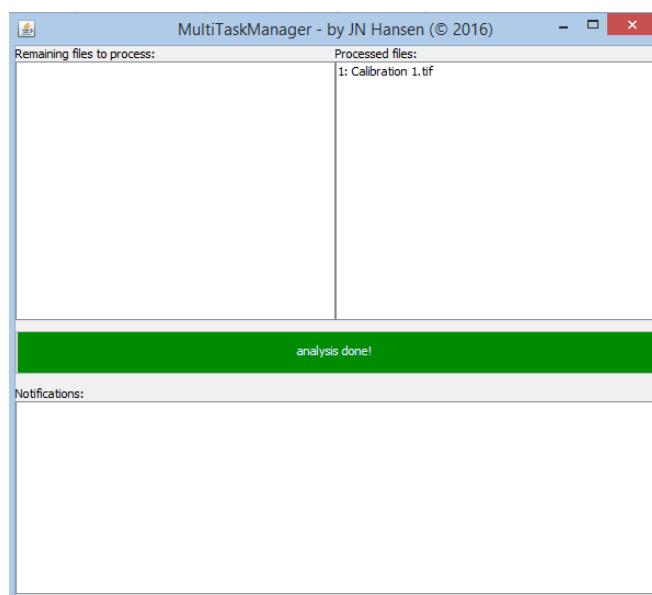
More information, license notes, credits: <https://github.com/hansenjn/MultifocalImaging-AnalysisToolbox>



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

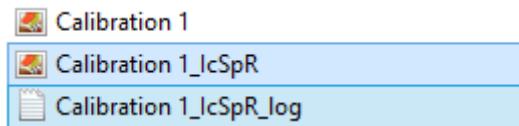


- To avoid any processing errors, don't touch the computer while processing. Some images might pop up and be hidden again during registration. Wait until processing is done.

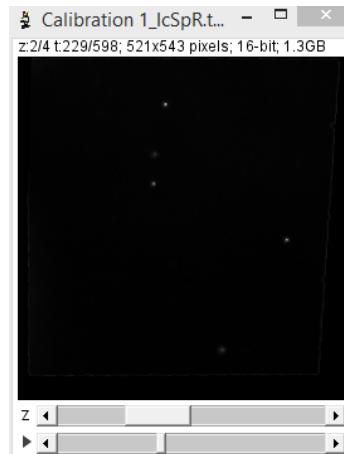


More information, license notes, credits: <https://github.com/hansenjn/MultifocalImaging-AnalysisToolbox>

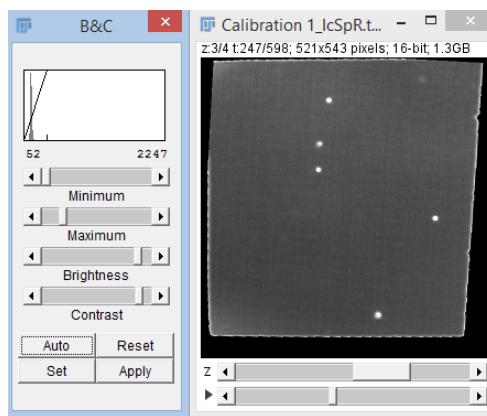
- At the location where the template files were saved, the output files will be saved. They receive an additional ending (_IcSpR).



- 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”.

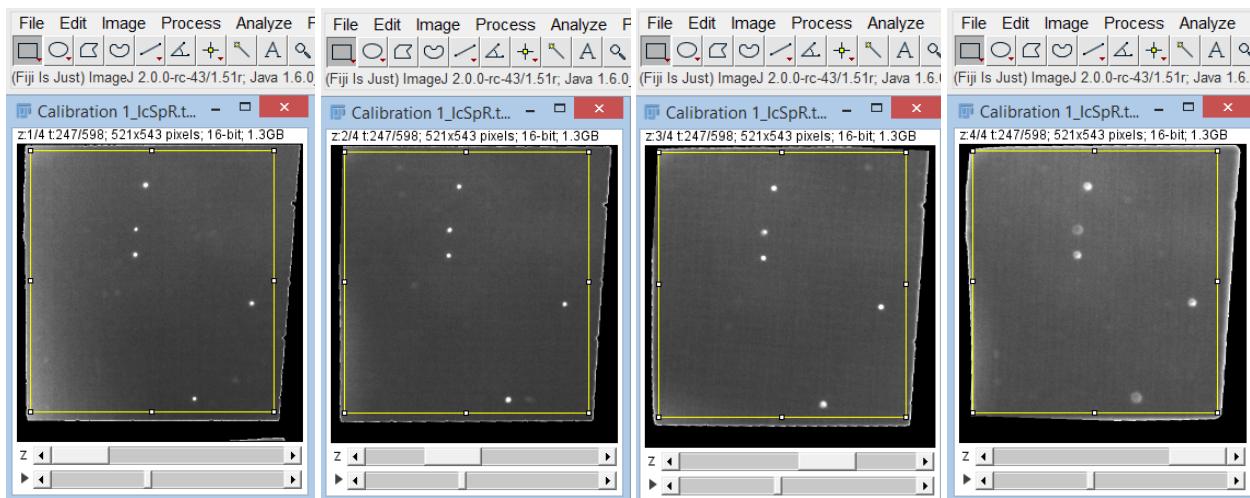


- Before analysis of the IcSpR image, crop the image to regions that are depicted in all four plane images:
 - Adapt the display range so that you can see the borders of the planes: Image > Adjust > Brightness/Contrast, drag down the maximum so that edges become apparent in the image

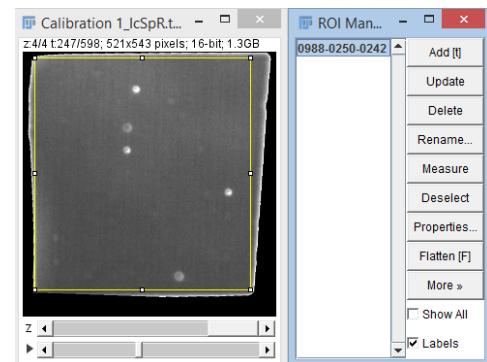


- Select the Rectangle tool in the ImageJ bar and draw a selection that is inside all planes and excludes all black areas. Check the different planes by scrolling with the mouse wheel and adapting the selection.

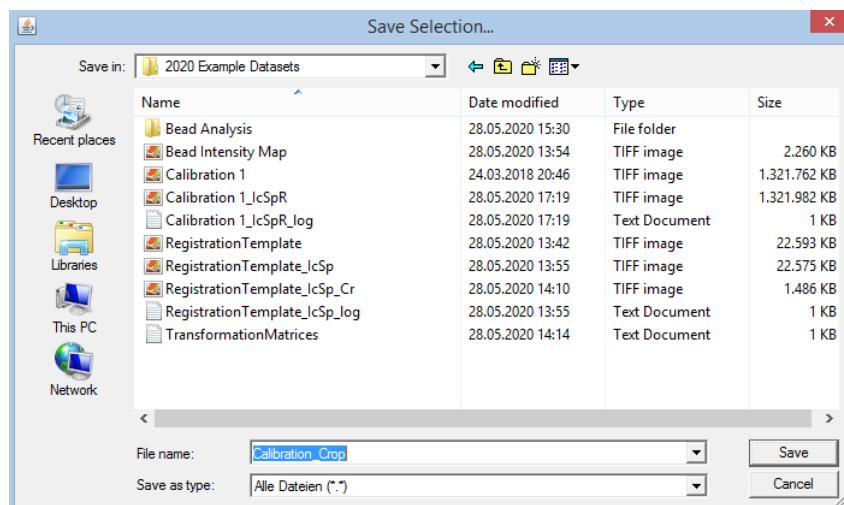
More information, license notes, credits: <https://github.com/hansenjn/MultifocalImaging-AnalysisToolbox>



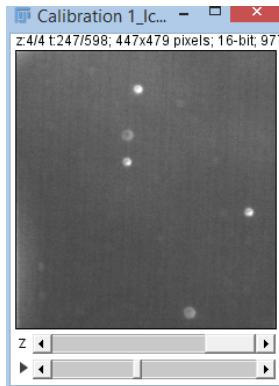
- You may save the selection for later use / reproduction of the image processing
 - Transfer the selection to the ROI Manager by pressing "t"



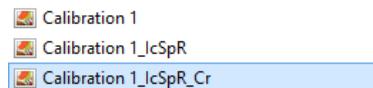
- Select the ROI in the ROI Manager and save it via More >> Save.



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



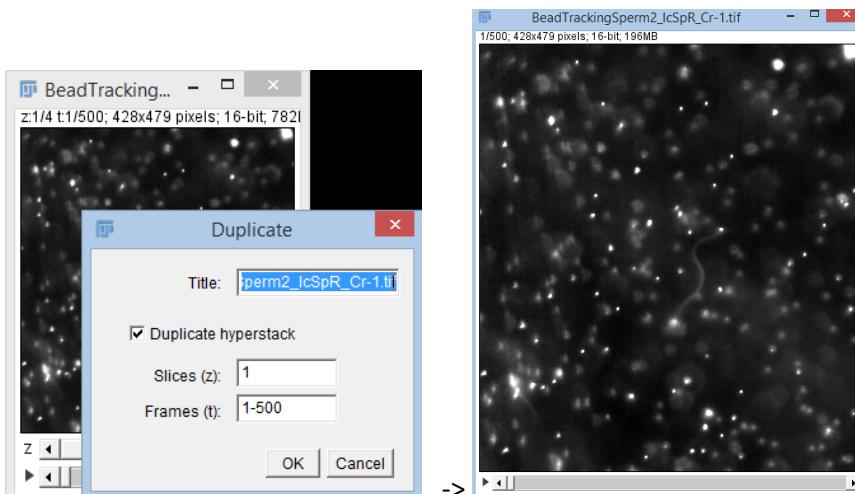
- Save the stack with the ending “_cr.tif” to your directory: File > Save As > Tiff...



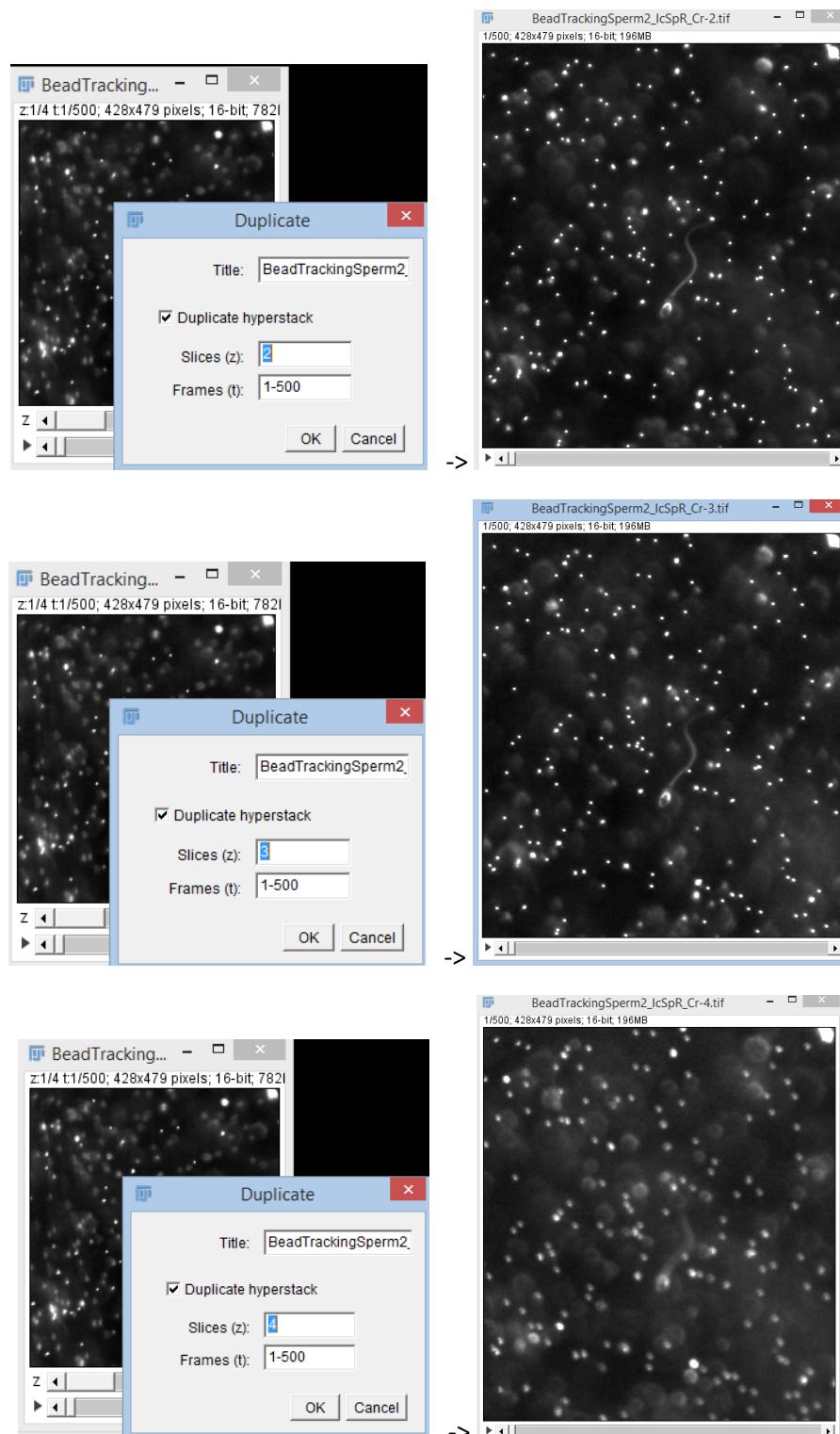
Background reduction for images recorded for bead tracking or sperm tracking

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 “...IcSpR_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).

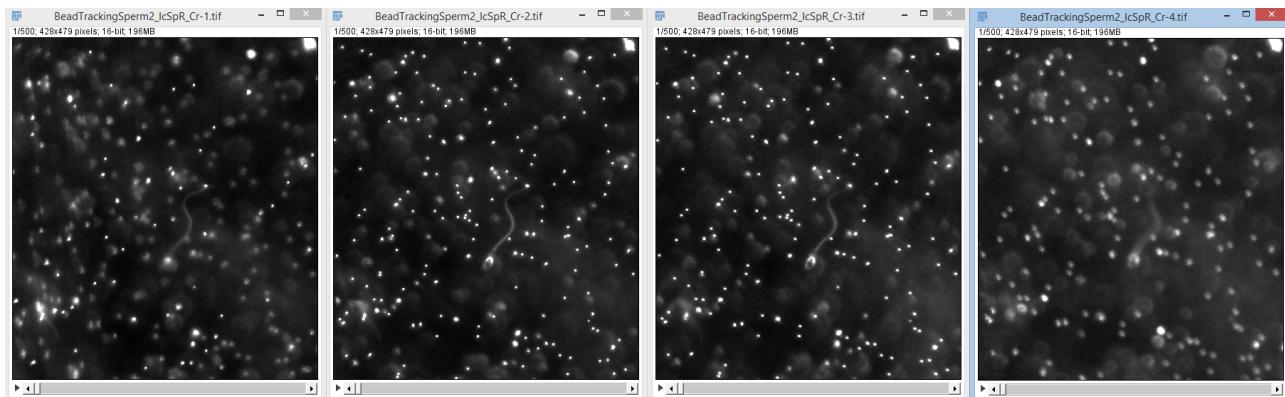


More information, license notes, credits: <https://github.com/hansenjn/MultifocalImaging-AnalysisToolbox>

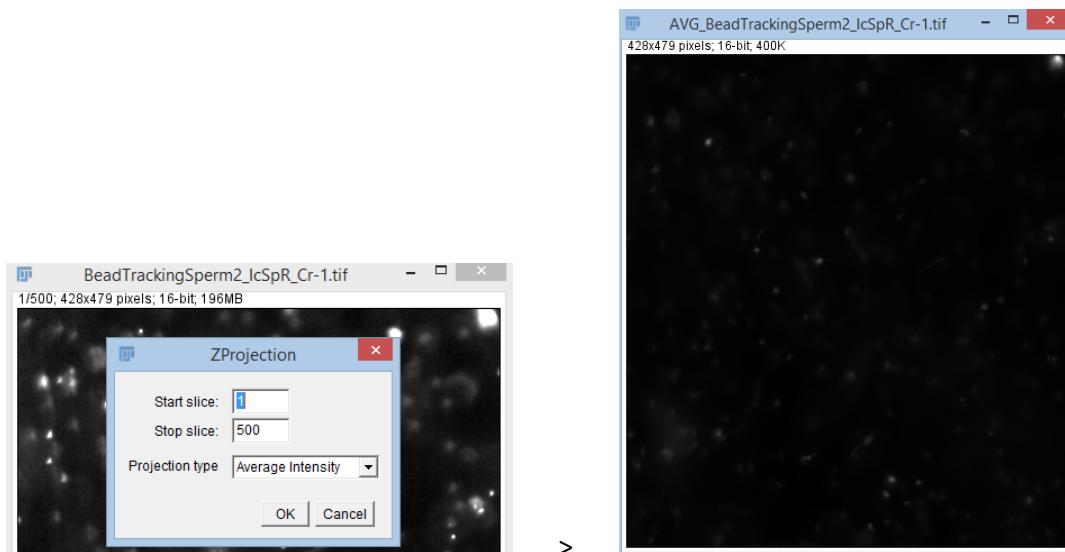


- Afterwards you should have an image for each of the four planes open in ImageJ:

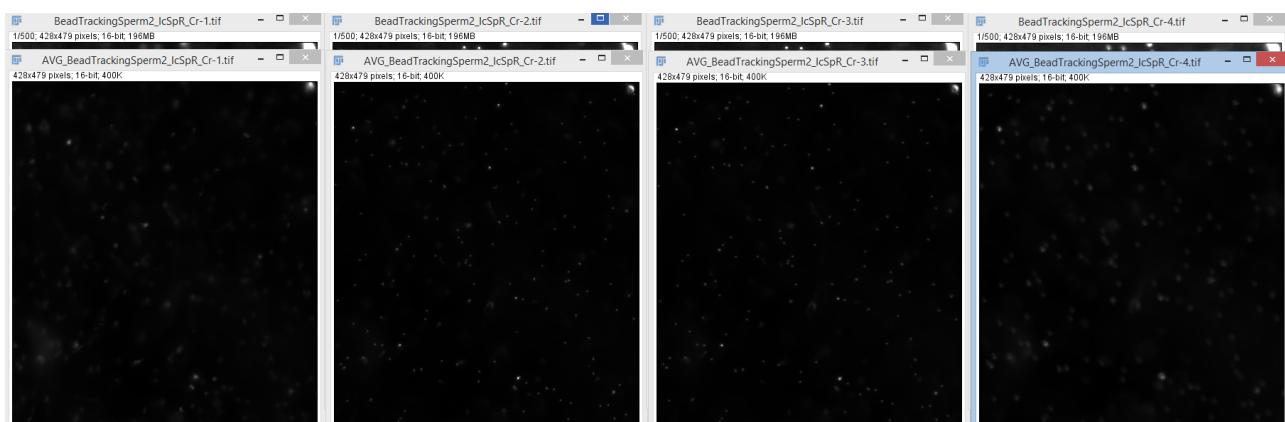
More information, license notes, credits: <https://github.com/hansenjn/MultifocalImaging-AnalysisToolbox>



- 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:

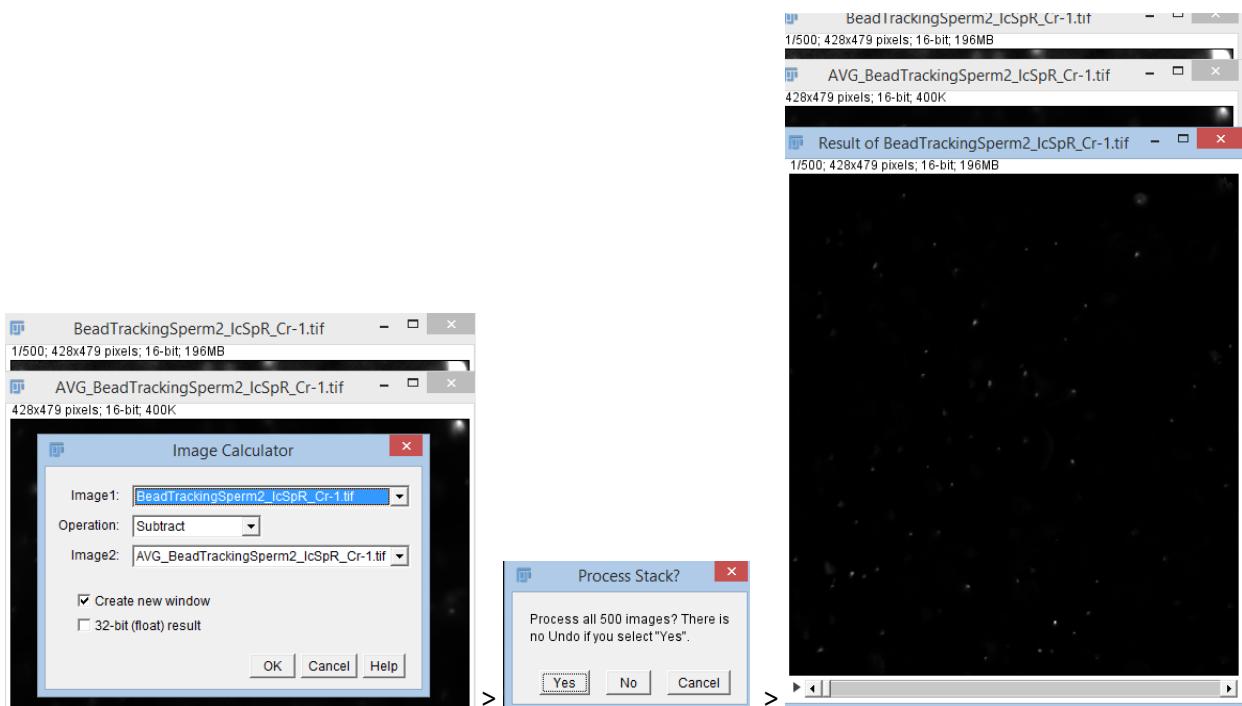


- Afterwards you should have an AVG projection for each plane image'

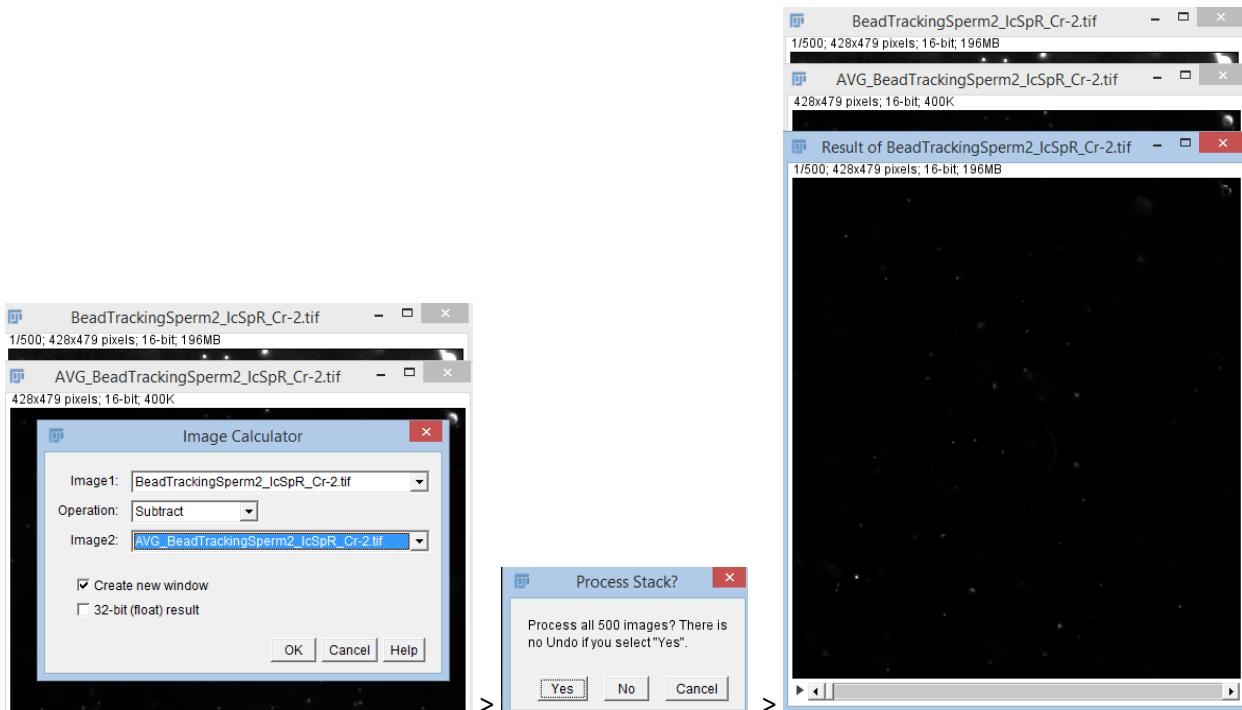


- For each plane image, subtract the respective AVG_... projection image from the image series: Process > Image Calculator, select the image series as Image 1 and the respective AVG projection image as Image 2, press OK, press Yes in the upcoming “Process Stack?” dialog.
 - Plane 1:

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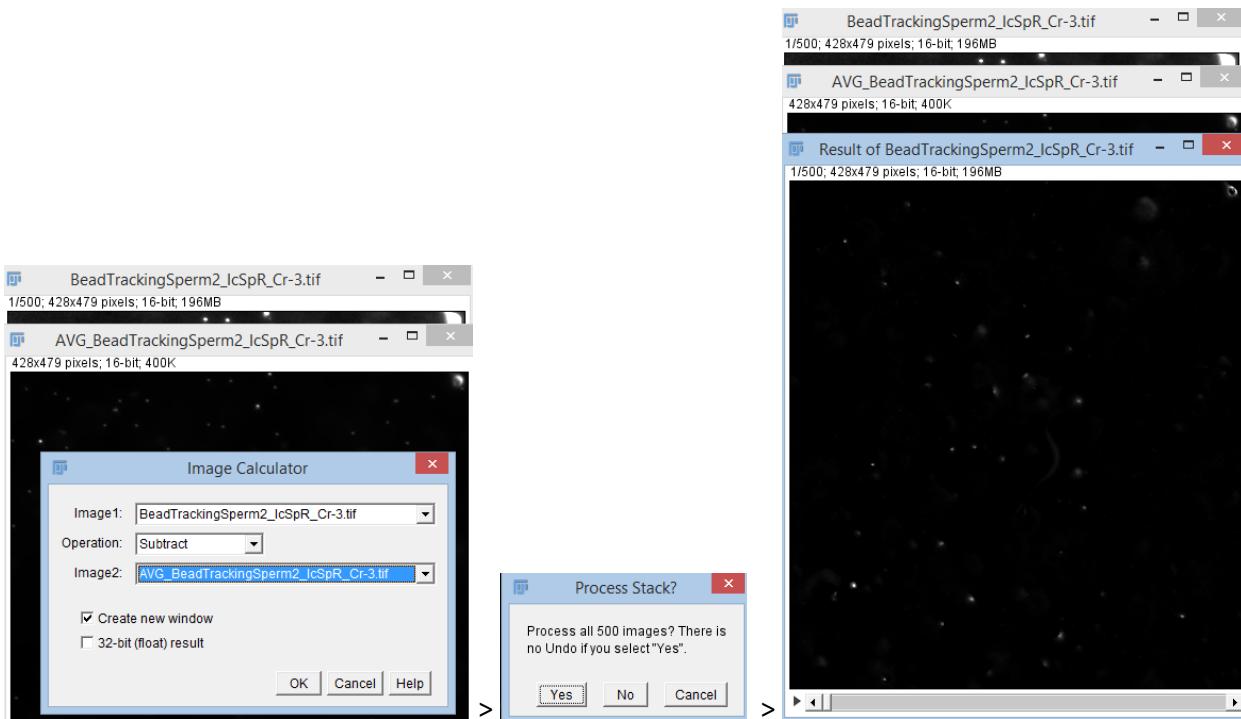


○ Plane 2

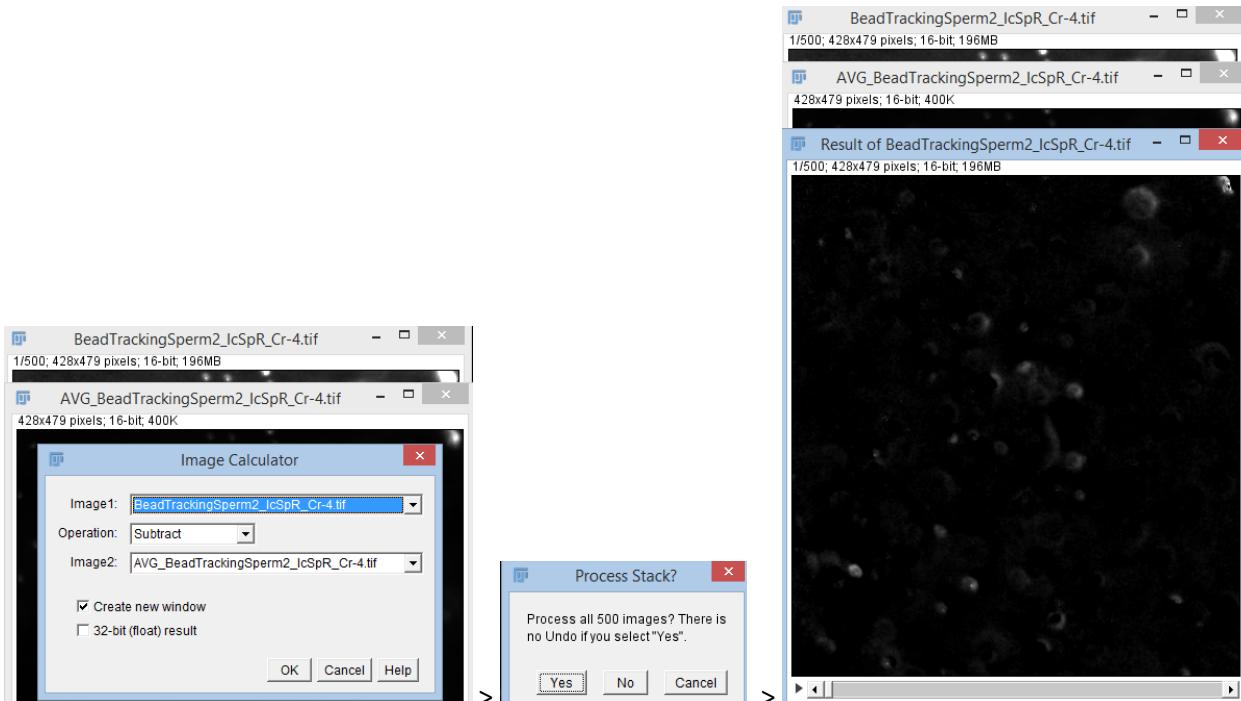


○ Plane 3

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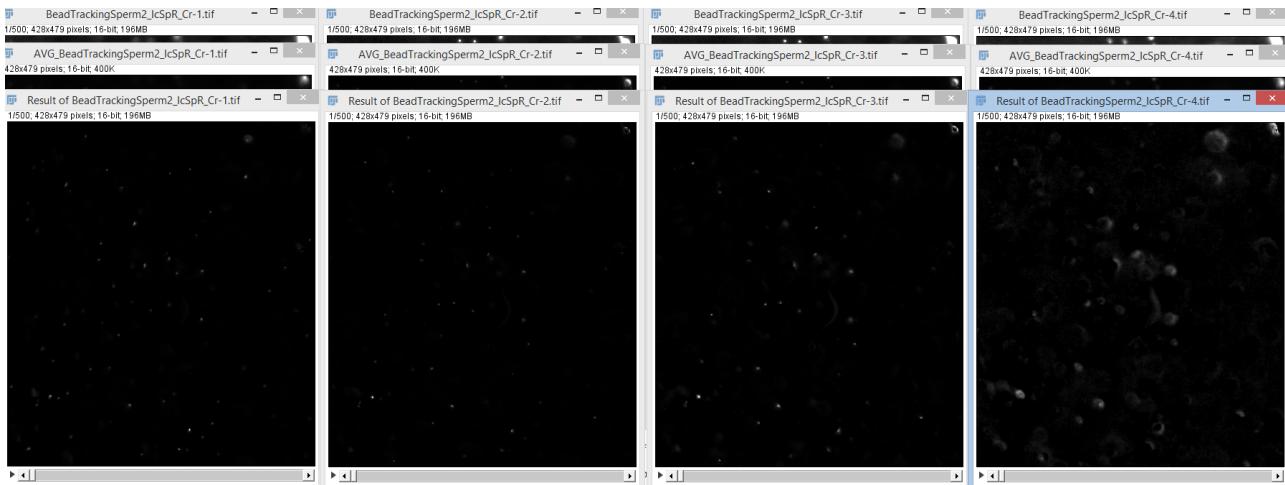


- **Plane 4**

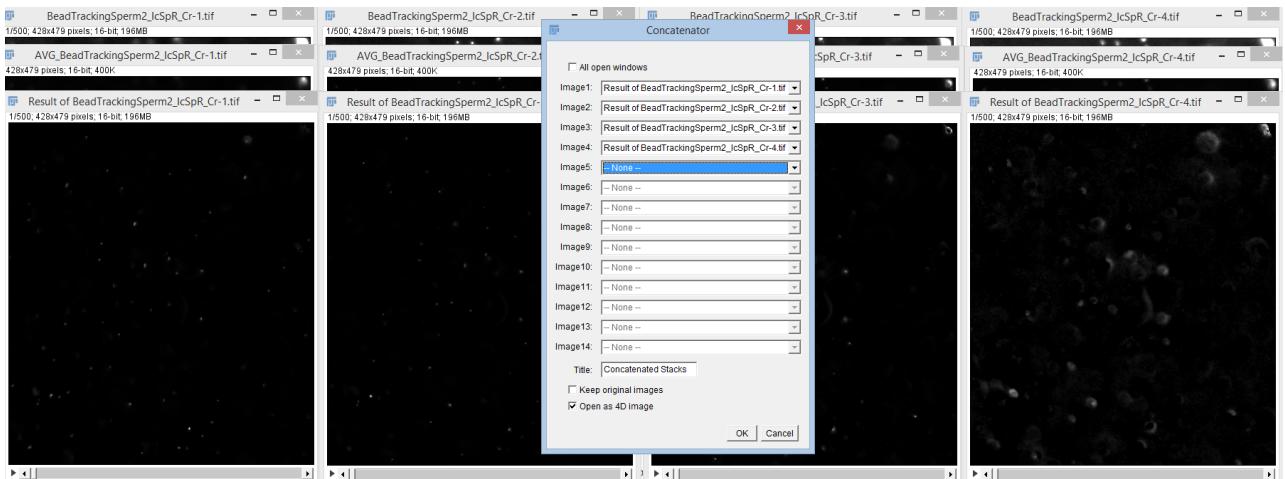


- You should end up with a subtracted image (window title starting with “Result of ...”) for each plane:

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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:

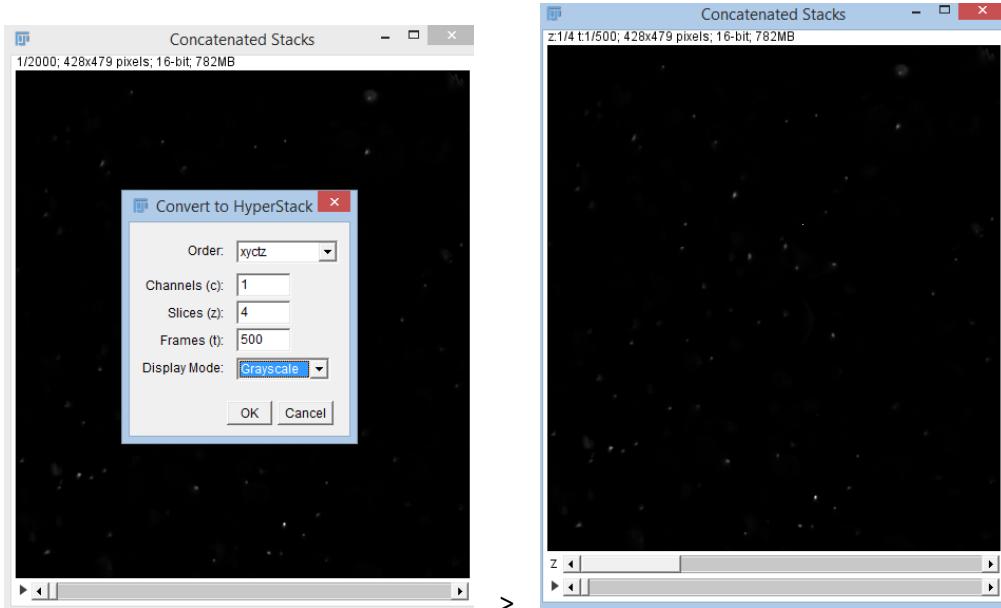


- Eventually the “open as 4D image” function might not work and you will end up with a 1-dimensional stack (the stacks for all four planes are then put into one dimension after each other):

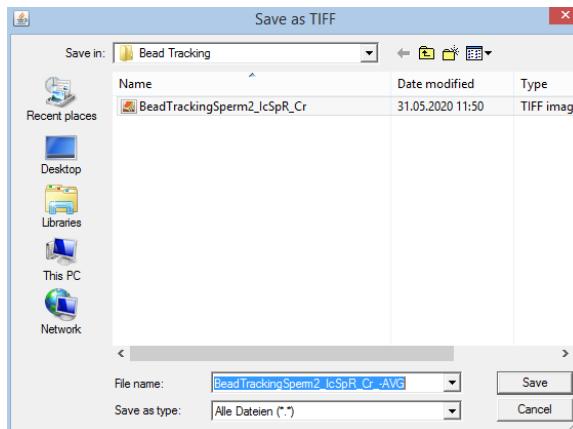


More information, license notes, credits: <https://github.com/hansenjn/MultifocalImaging-AnalysisToolbox>

- 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)

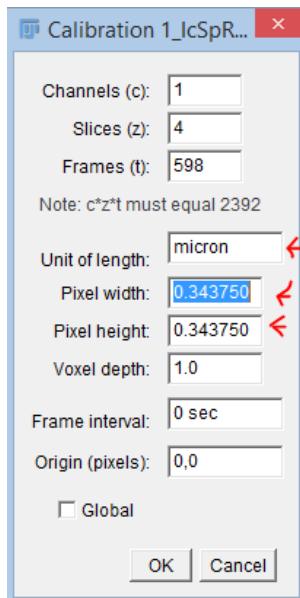
To determine a calibration Look-Up-Table (LUT) you need to record a z-stack with a defined gap between different recorded z-positions, e.g. set by a piezo, through non-moving beads with your multifocal imaging setup. Before performing the steps described in this chapter, make sure the image was preprocessed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

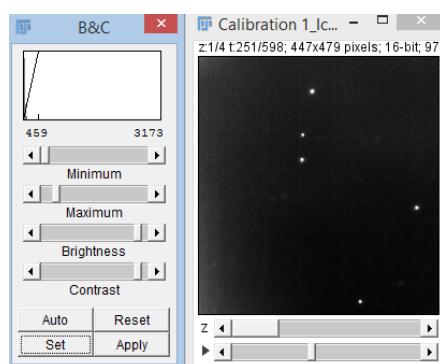
- Create an empty text file with name “<your image’s name>_spots.txt” and open it so that you can note down the particle positions
- Open the image for which you want to note done particle positions in ImageJ
- Make sure the image is correctly calibrated: Image > Properties; If the information provided 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

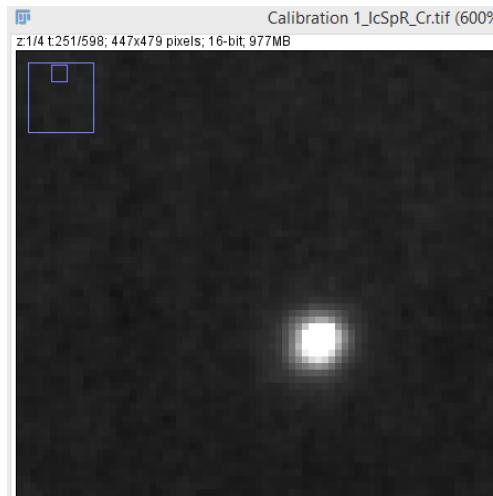


- Eventually you may need to adapt the Brightness and Contrast of the image to see the beads: Image > Adjust > Brightness/Contrast, drag down the maximum

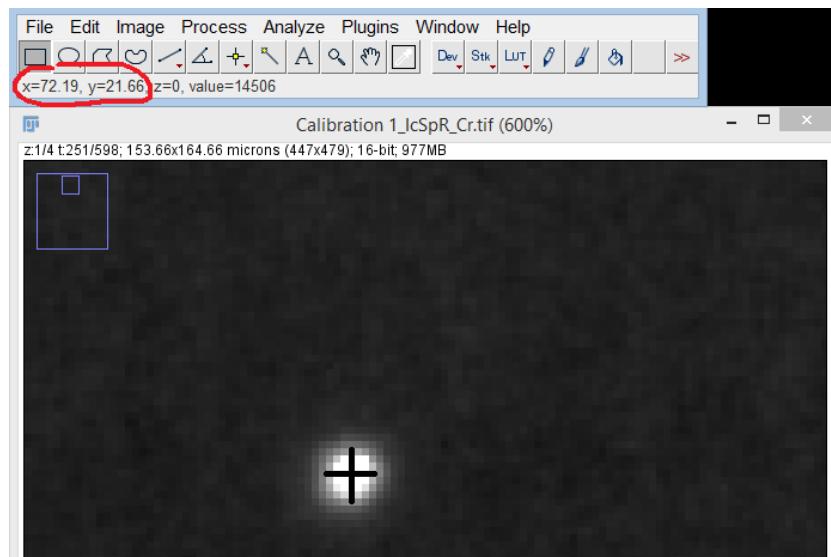


- Zoom into a bead by pressing Ctrl and using the mouse wheel

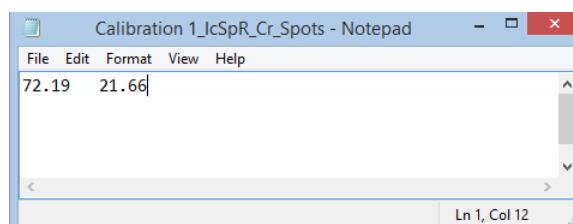
More information, license notes, credits: <https://github.com/hansenjn/MultifocalImaging-AnalysisToolbox>



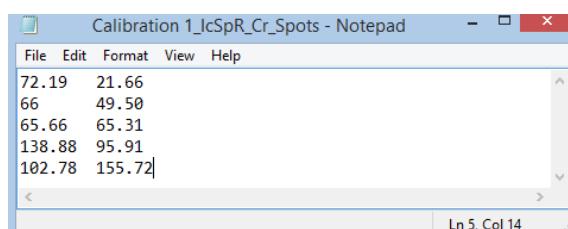
- Hover over the center of a bead to see its calibrated x and y position in the status bar of the ImageJ panel (encircled in red on the image below):



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

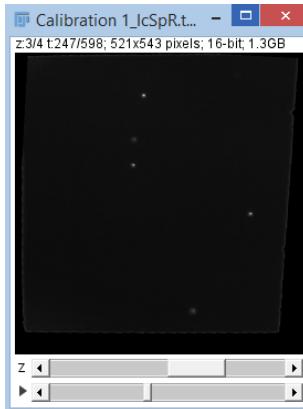


- Zoom into other beads in the image and also note down the coordinates in the text file in additional rows. Each bead position is noted in a separate row.

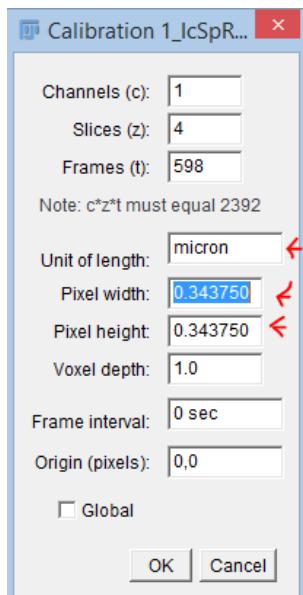


More information, license notes, credits: <https://github.com/hansenjn/MultifocalImaging-AnalysisToolbox>

- 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.

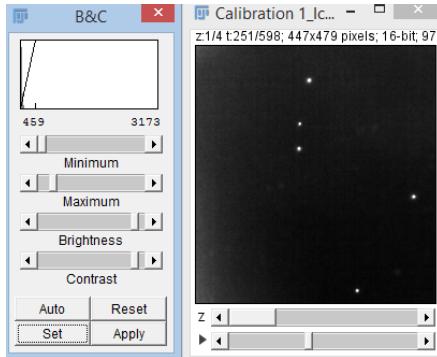


- Change the stack position with the bars on bottom of the image until you can clearly see the beads you aim to analyze

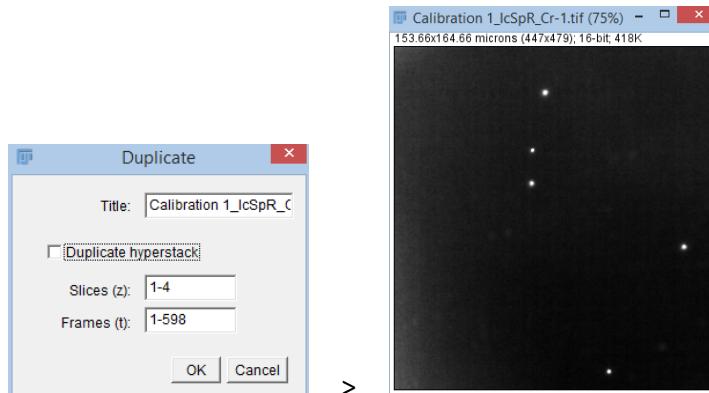


More information, license notes, credits: <https://github.com/hansenjn/MultifocalImaging-AnalysisToolbox>

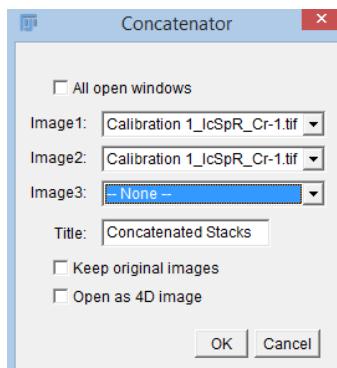
- Eventually you may need to adapt the Brightness and Contrast of the image to see the beads: Image > Adjust > Brightness/Contrast, drag down the maximum



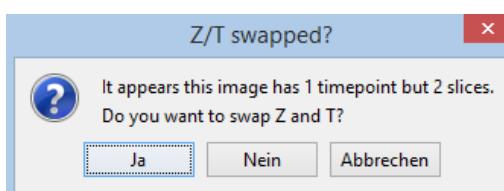
- Duplicate that plane image to use it for analysis by TrackMate: Image > Duplicate, unselect “Duplicate hyperstack”, press OK



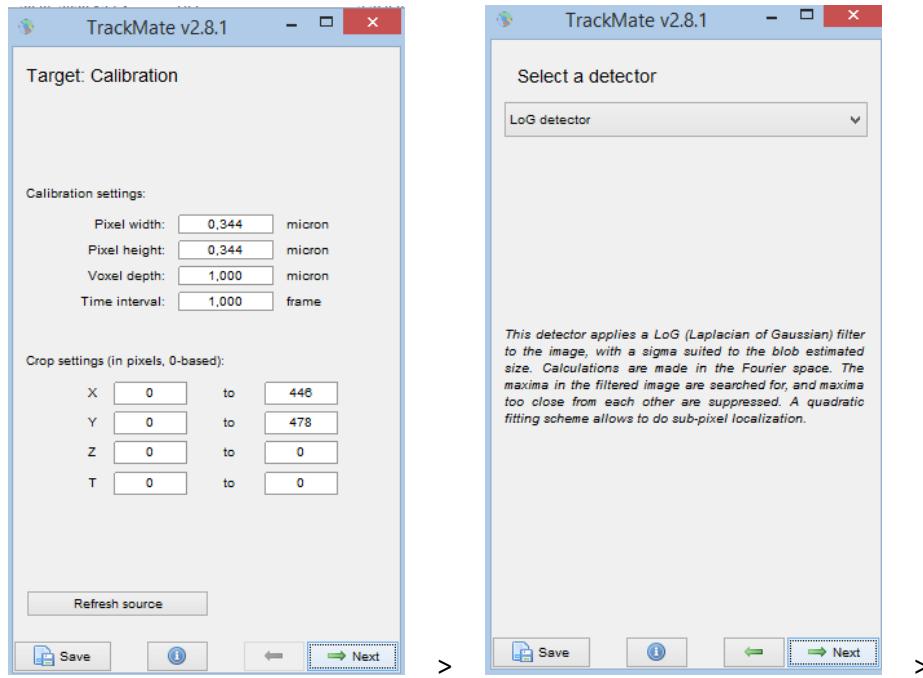
- Create a stack of twice this image: Image > Stacks > Tools > Concatenate..., select the duplicated image as Image1 and as Image2:



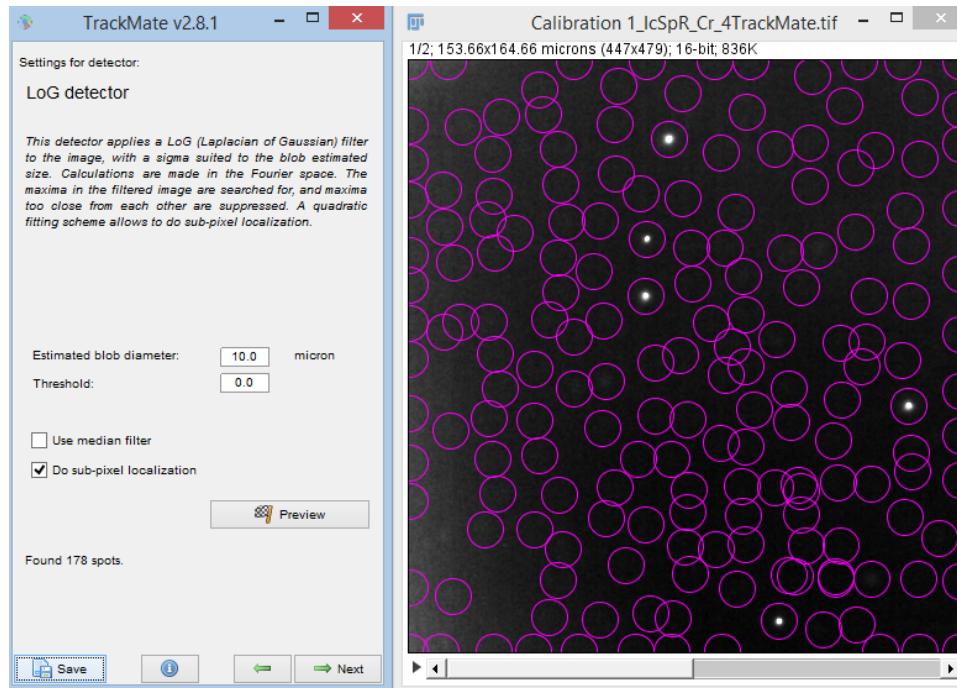
- Save the stack with the ending “_4TrackMate.tif” to your directory: File > Save As > Tiff...
- Launch TrackMate: Plugins > Tracking > TrackMate; if a dialog pops up asking you to swap Z and T, confirm by pressing Yes (or “Ja” if you have a German computer):



- A dialog pops up - press Next:

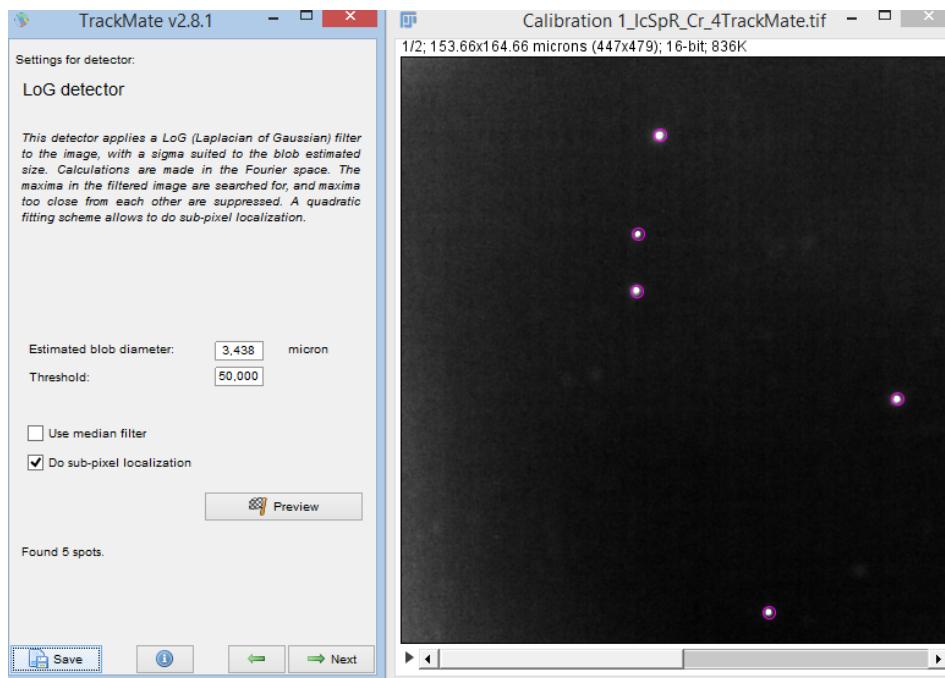


- Press Next and then "Preview" to see the positions that will be detected as a particle (will be encircled in purple)

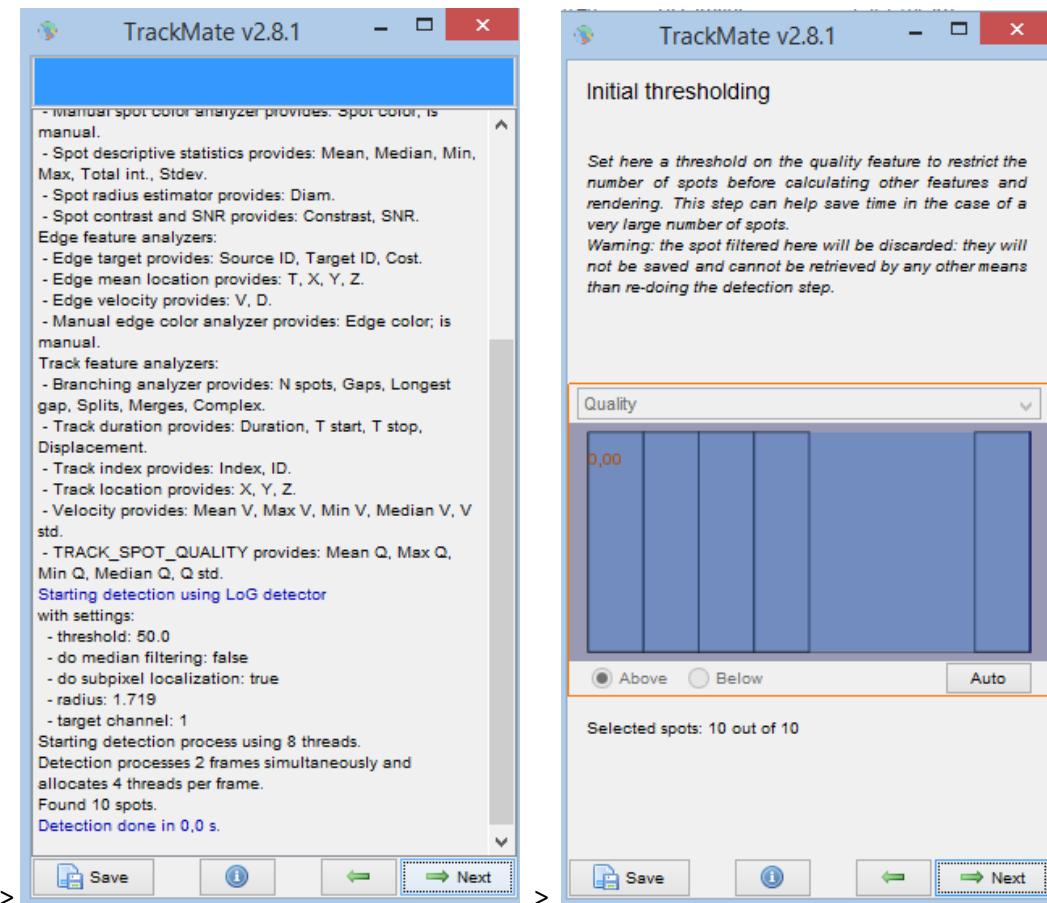


- Adapt the blob diameter and the threshold until the detection is correct.

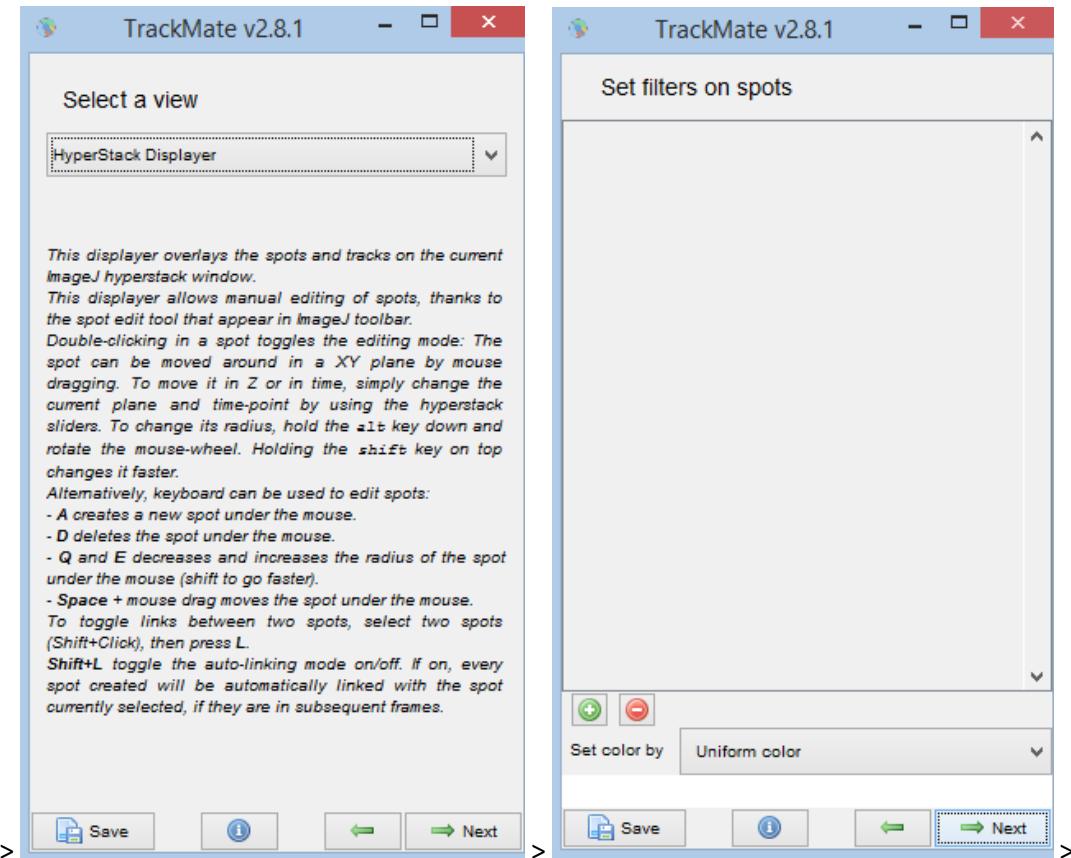
More information, license notes, credits: <https://github.com/hansenjn/MultifocalImaging-AnalysisToolbox>



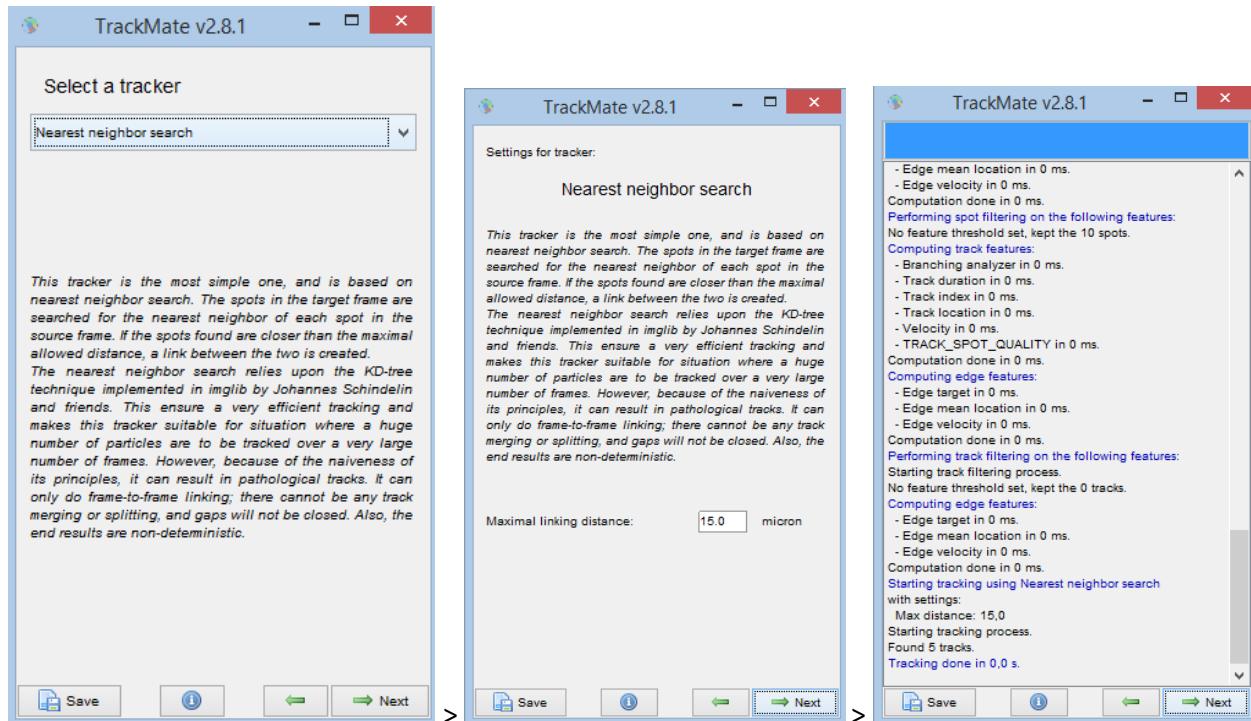
- Click five times Next until you reach a dialog showing “Select Tracker”:



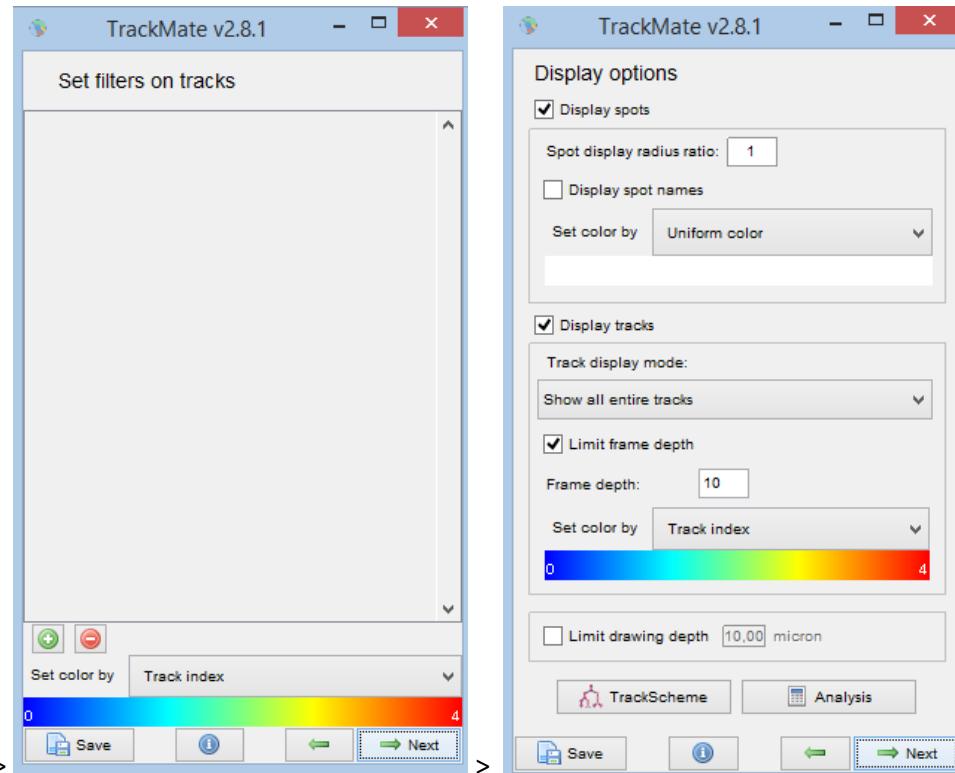
More information, license notes, credits: <https://github.com/hansenjn/MultifocalImaging-AnalysisToolbox>



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



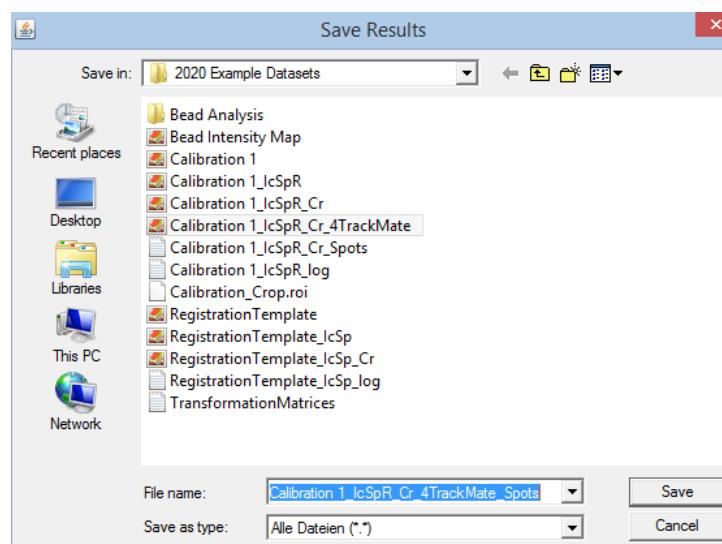
More information, license notes, credits: <https://github.com/hansenjn/MultifocalImaging-AnalysisToolbox>



- 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
1 Track_0	2	0	0	1 (ID387 : ID389)	0	387	389	1 ID387	387	0	460.24277
2 Track_1	2	0	0	2 (ID386 : ID390)	1	386	390	2 ID389	389	0	460.24277
3 Track_2	2	0	0	3 (ID385 : ID393)	2	385	393	3 ID386	386	1	396.92221
4 Track_3	2	0	0	4 (ID384 : ID392)	3	384	392	4 ID390	390	1	396.92221
5 Track_4	2	0	0	5 (ID388 : ID391)	4	388	391	5 ID385	385	2	425.63721

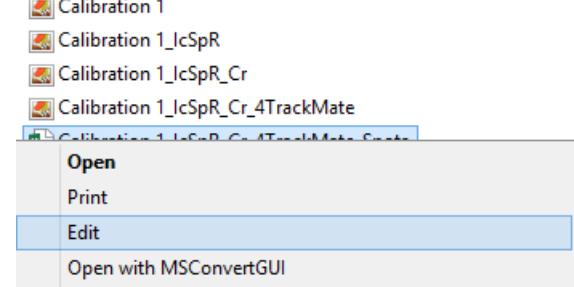
- Close the windows “Track statistics” and “Links in tracks statistics” without saving, select the window “Spots in tracks statistics” and save it as a .csv file: File > Save As ...



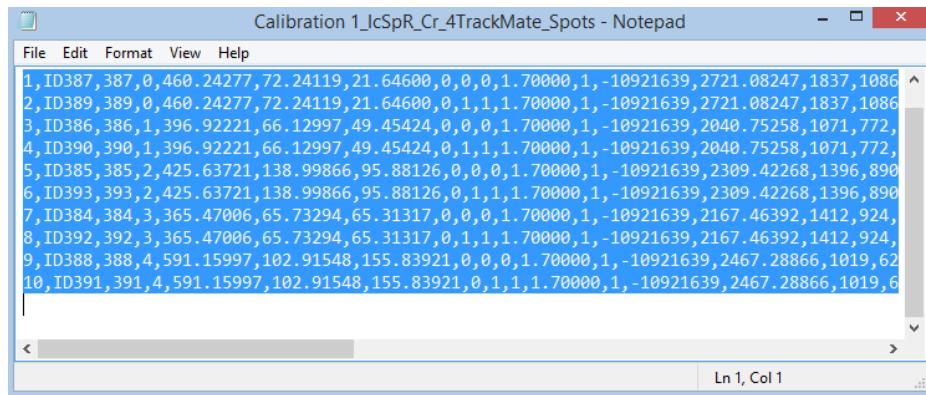
More information, license notes, credits: <https://github.com/hansenjn/MultifocalImaging-AnalysisToolbox>

- 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:

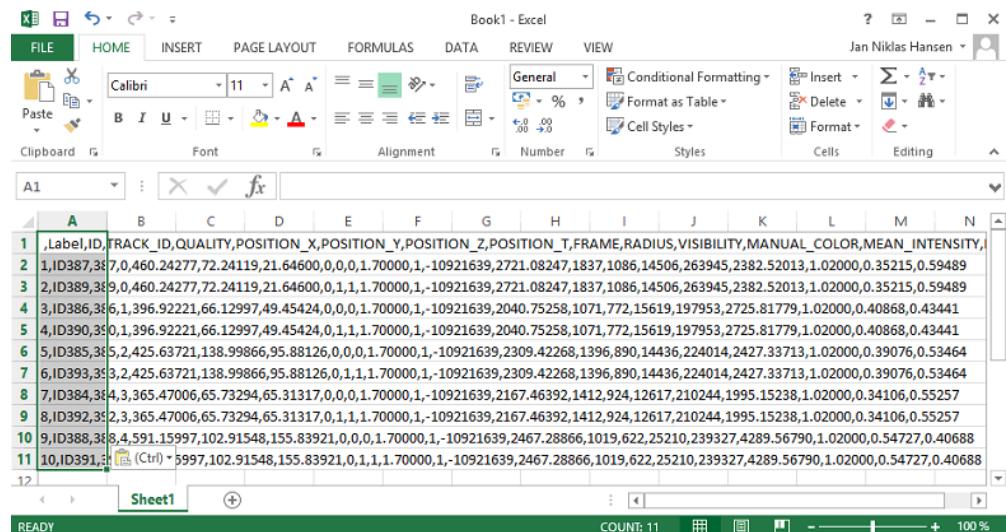
- Open the saved .csv file with a text editor



- Mark all and press Ctrl + C:

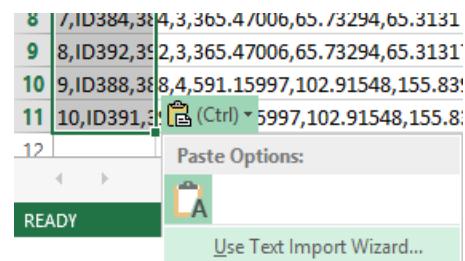


- Open Excel or a similar table calculation software and press Ctrl + V

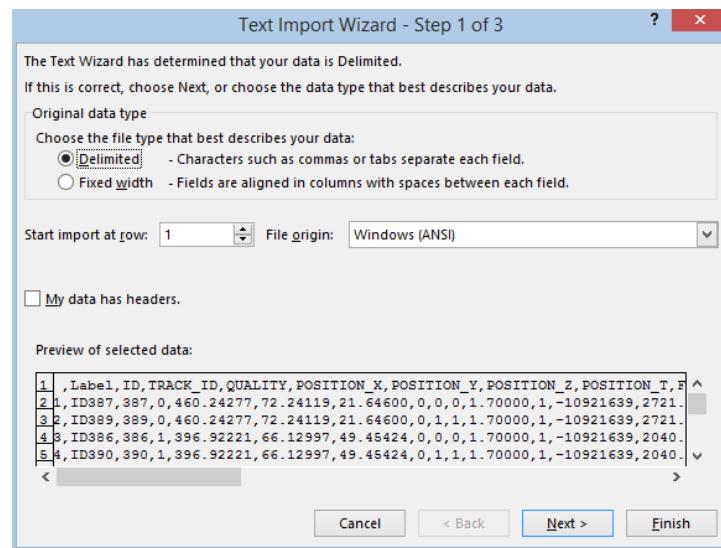


- Click on the small Ctrl button on the bottom and select "Use Text Import Wizard..."

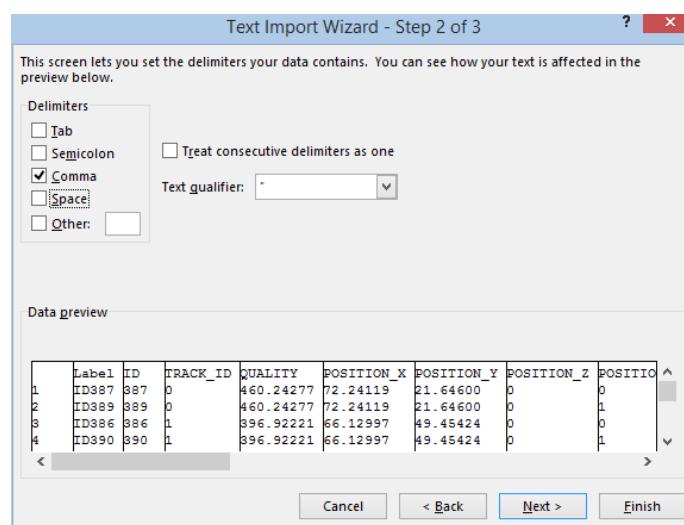
More information, license notes, credits: <https://github.com/hansenjn/MultifocalImaging-AnalysisToolbox>



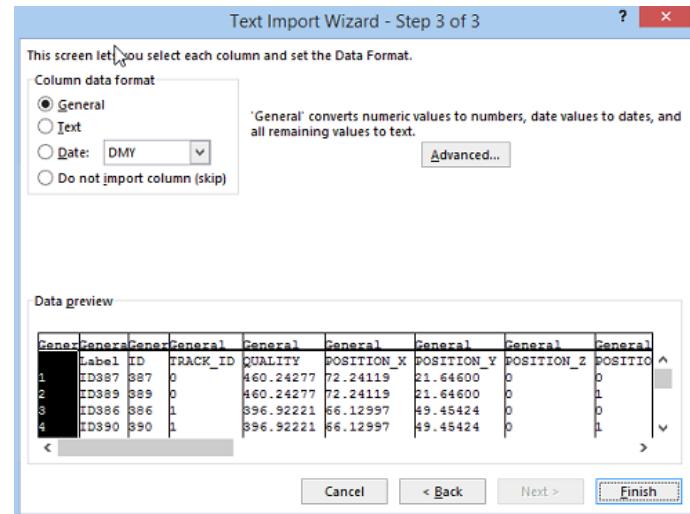
- Select Delimited and press Next



- Select Comma (see below) and press Next



- Press Finish



- Select all (usually all is already selected)

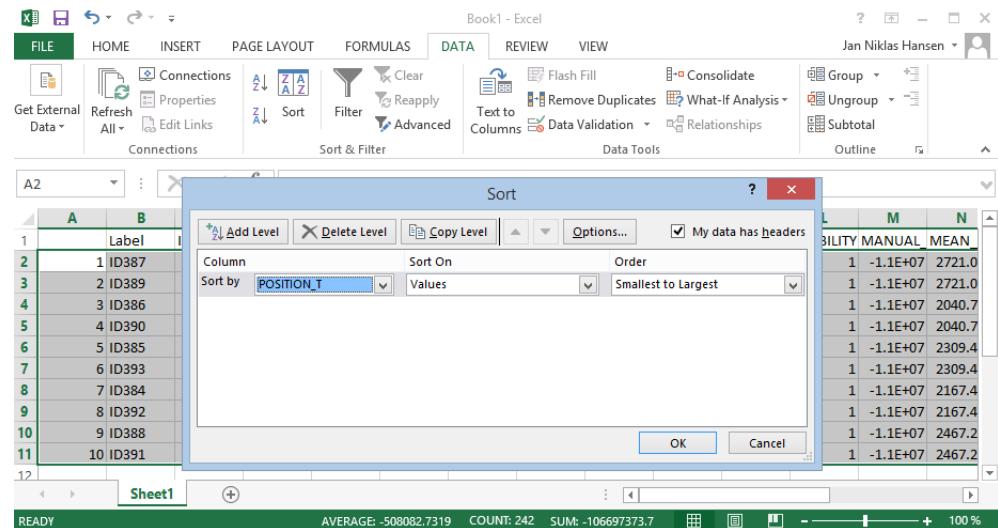
A	B	C	D	E	F	G	H	I	J	K	L	M	N
1	Label	ID	TRACK_ID	QUALITY	POSITION_X	POSITION_Y	POSITION_Z	POSITION_T	FRAME	RADIUS	VISIBILITY	MANUAL_MEAN	
2	1	ID387	387	0	460.2428	72.24119	21.646	0	0	0	1.7	1	-1.1E+07 2721.0
3	2	ID389	389	0	460.2428	72.24119	21.646	0	1	1	1.7	1	-1.1E+07 2721.0
4	3	ID386	386	1	396.9222	66.12997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

- Select POSITION_T as "Sort by" and press OK:

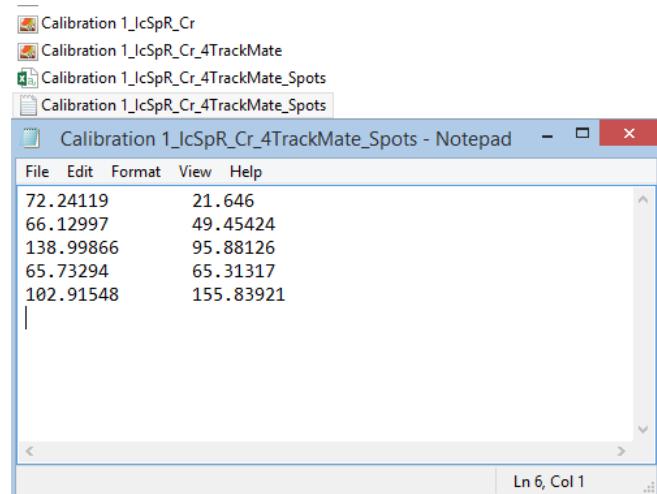
More information, license notes, credits: <https://github.com/hansenjn/MultifocalImaging-AnalysisToolbox>



- 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:

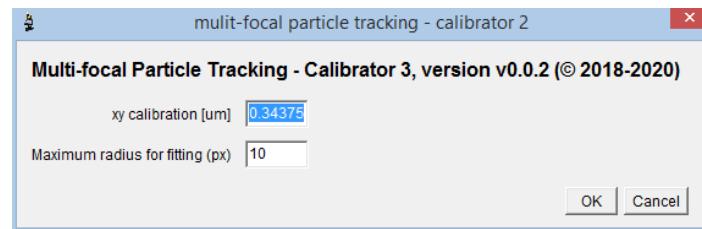


- Save the text file.

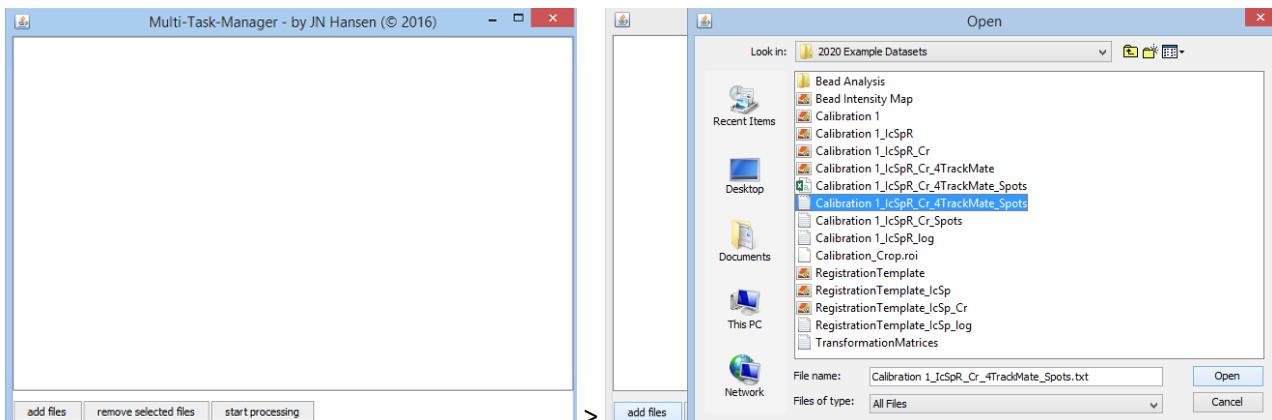
Now that a file that contains the positions to be analyzed has been created, analysis of the bead width at different z positions with the MultiFocalParticleTracker-Calibrator plugin can be conducted:

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

- Open ImageJ
- 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 μm / px
 - Enter the radius that you want to consider for estimating the width of the bead.
 - If the radius is small beads can be 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

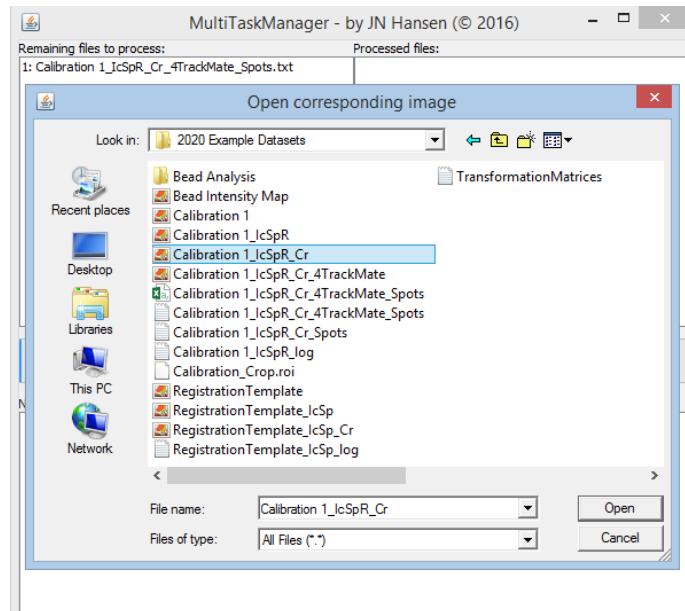


- A dialog pops up: add the text file containing the bead positions and press “start processing”:

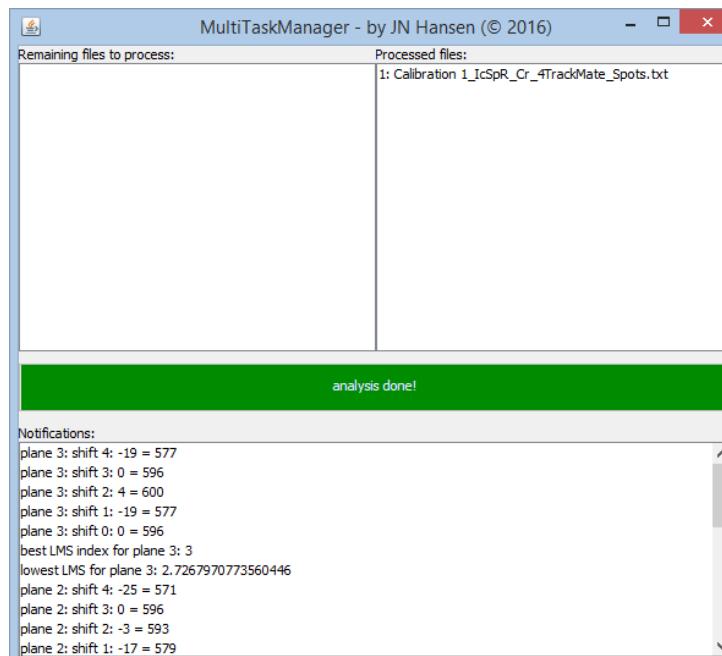


- A dialog pops up requesting to open the corresponding image. Select the corresponding image and press Open:

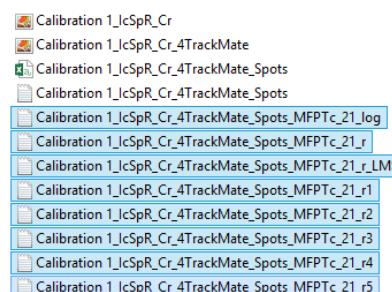
More information, license notes, credits: <https://github.com/hansenjn/MultifocalImaging-AnalysisToolbox>



- Wait until MultiTaskManager states “analysis done!”

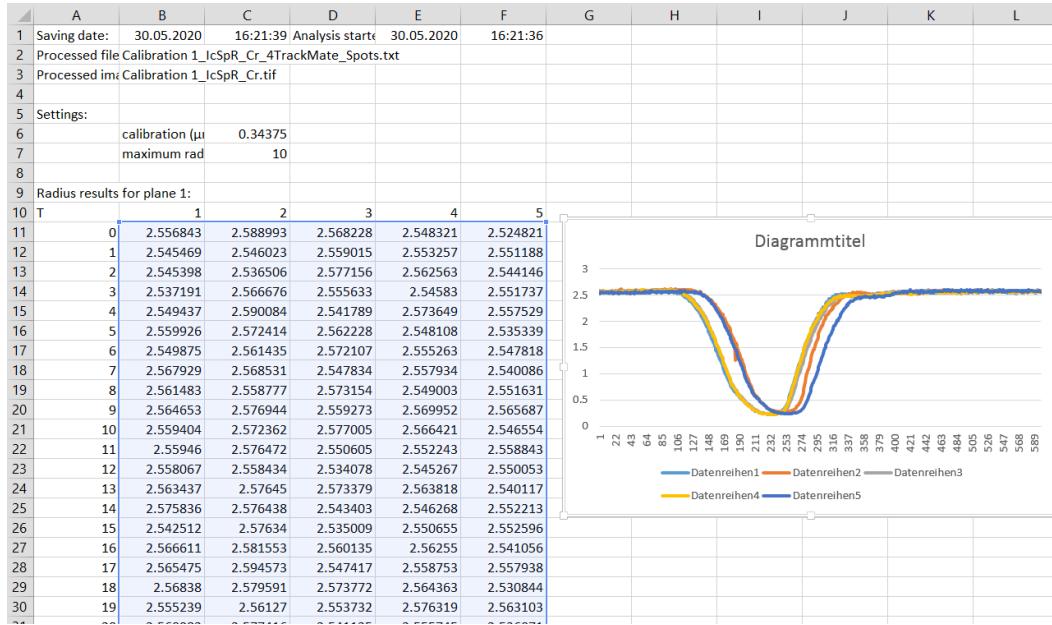


- The plugin has saved new files to the folder where the text file with bead position was saved. They all contain an additional suffix (“_MFPTc_<number>_”) and specific file endings.



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

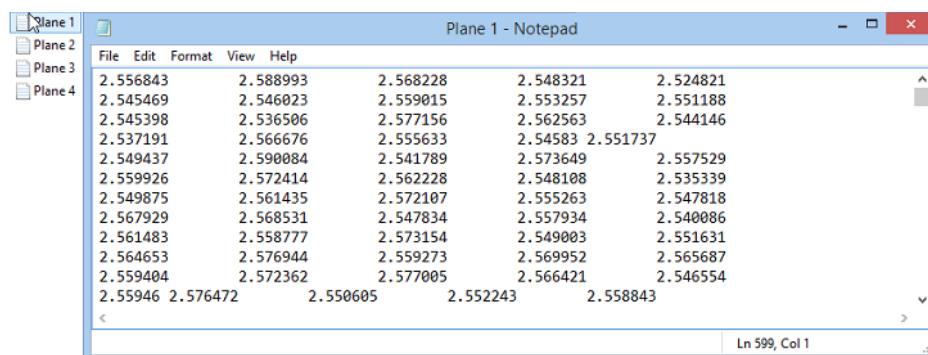
- Open the file with ending "..._r.txt" and copy the content e.g. to excel to investigate the determined widths:



- 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:

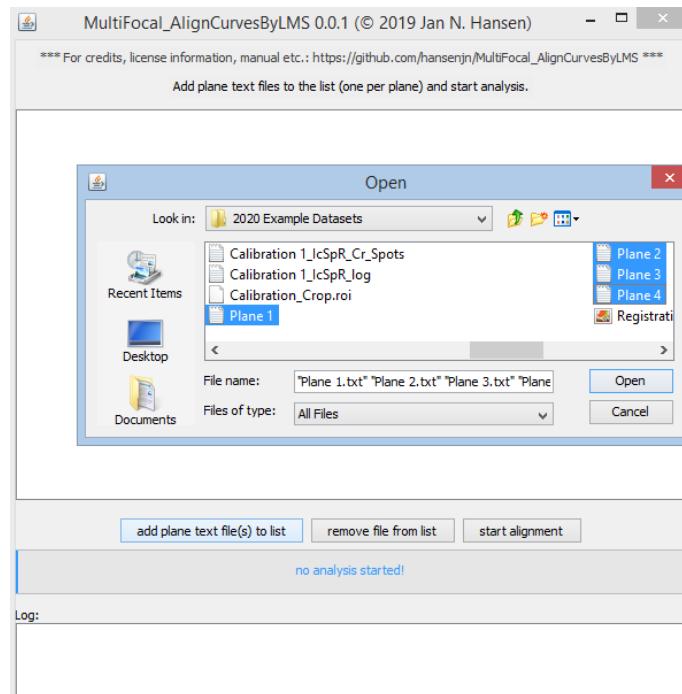
- Generate 4 text files, entitled e.g.:
 - Plane 1.txt
 - Plane 2.txt
 - Plane 3.txt
 - Plane 4.txt
- Copy the pure width values as selected in the image above for each plane to the corresponding text file, e.g.



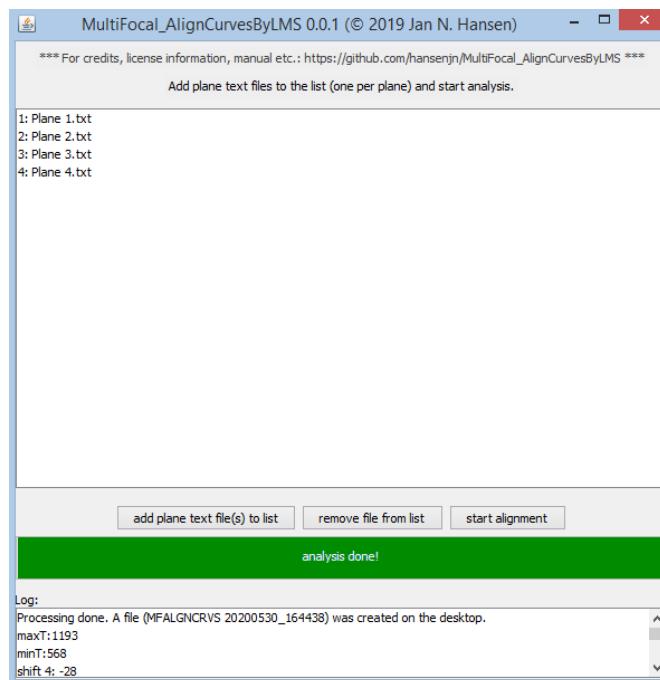
- In each file, each column must contain the width values for one bead at different z-positions
 - Do not include a column with slice positions!
 - Do not include a first row with bead labels!

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

- 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).
- Add the plane text files to the list: press “add plane text file(s)”, select the text files, press Open.

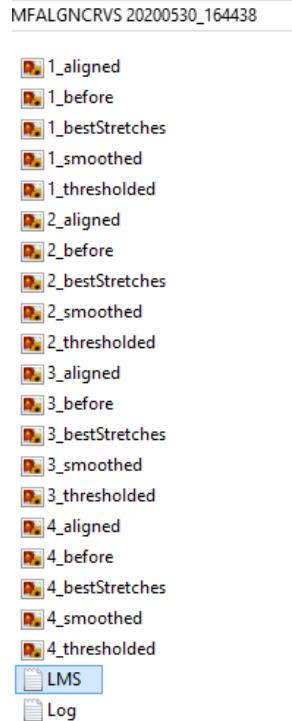


- Press start alignment and wait until analysis is done. During analysis, the plugin shifts the curves of the beads along each other (but always with the same shift in all planes) and determines the LMS between the curves across all planes. The shift with the minimum LMS result is selected and a table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

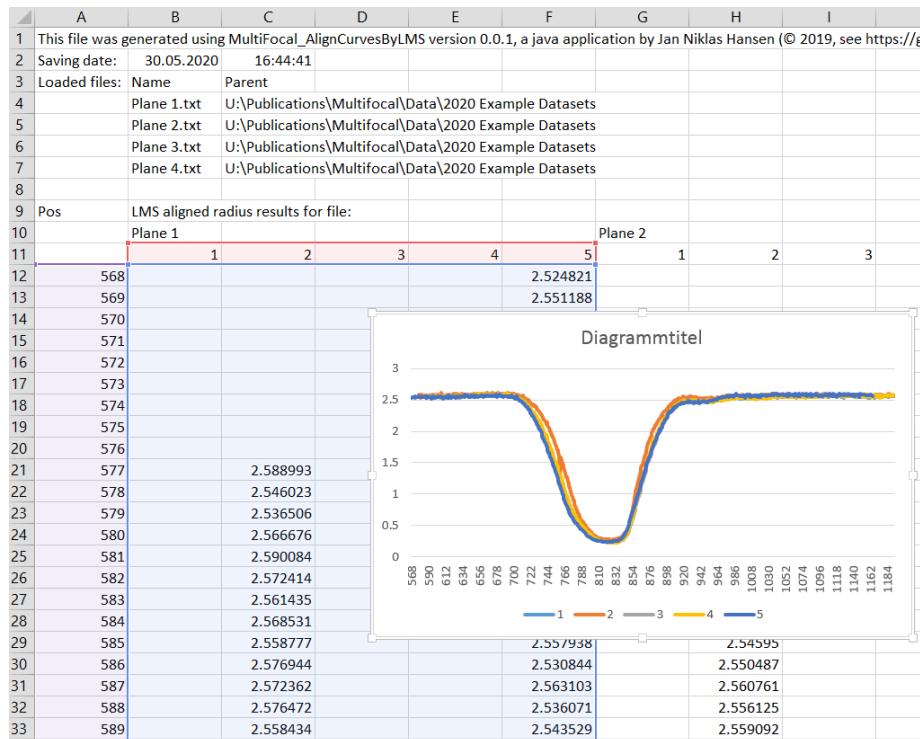


More information, license notes, credits: <https://github.com/hansenjn/MultifocalImaging-AnalysisToolbox>

- 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

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

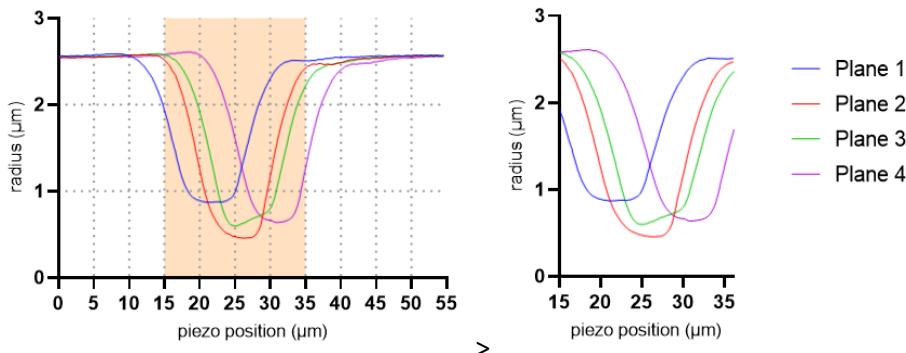
- Thereby, create a table indicating the z-position and the four columns (one for each plane)

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	2.556867	
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	2.551887	2.544526	2.5612825	

- Eventually, use a smoothing algorithm to remove noise
- **New from 13.05.2021 on:** For each plane, also determine the standard deviation of the different widths at each position, creating a table indicating the z-position in the first column and the standard deviation for the planes in the next columns:

A	B	C	D	E	
1	z	Plane 1	Plane 2	Plane 3	Plane 4
2	0	0.018462	0.013187	0.021217	0.016443
3	0.1	0.012696	0.017545	0.024123	0.016578
4	0.2	0.008998	0.017822	0.018013	0.013819
5	0.3	0.010936	0.017751	0.012494	0.021521
6	0.4	0.008064	0.018952	0.010228	0.014914
7	0.5	0.015717	0.019413	0.014576	0.008445
8	0.6	0.015461	0.016947	0.020167	0.011106
9	0.7	0.024825	0.028079	0.015573	0.014783
10	0.8	0.017667	0.020519	0.016266	0.019393
11	0.9	0.010553	0.01958	0.014047	0.010104

- 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 ranges from the two tables, (1) z-position vs average bead width, (2) z-position vs standard deviation of bead width, into two text-files and save them as LUT.txt and SD_of_LUT.txt, respectively.

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

- Do not include a head / first row with information about the planes
- **LUT.txt file:** The first column represents the z-position, the second column represents the average width in plane image 1, the third column represents the average width in plane image 2, etc.

The screenshot shows a Windows Notepad window titled "LUT - Notepad". The content of the file is a tab-separated text table:

z	width1	width2	width3	width4
15	1.956610889	2.522831806	2.578865539	2.579702942
15.1	1.929122165	2.516855965	2.557505329	2.581174678
15.2	1.901283622	2.507740043	2.574346592	2.582006476
15.3	1.873375676	2.500305414	2.57419151	2.583375961
15.4	1.845233534	2.492493714	2.571141192	2.583646542
15.5	1.816167445	2.482251083	2.567202965	2.587025603
15.6	1.787747363	2.472277138	2.56547955	2.589025038
15.7	1.754649506	2.462622463	2.562444767	2.589635382
15.8	1.726293144	2.455792725	2.56106212	2.589926422
15.9	1.686639706	2.437798588	2.553272244	2.592566534
16	1.650279237	2.421443095	2.546030522	2.59383278
16.1	1.616108459	2.406960104	2.540217826	2.593917994
16.2	1.584845419	2.393243636	2.534553551	2.591626318

- **SD_of_LUT.txt file:** The first column represents the z-position, the second column represents the standard deviation of the width in plane image 1, the third column represents the standard deviation of the width in plane image 2, etc.

The screenshot shows a terminal window titled "SD_of_LUT.txt" containing the following tab-separated text table:

z	sd1	sd2	sd3	sd4
15	0.188245899	0.028153835	0.015443932	0.016234207
15.1	0.205246463	0.03506317	0.016843311	0.012057115
15.2	0.211911834	0.042217886	0.021595688	0.012658114
15.3	0.229150355	0.050336809	0.015556396	0.015611714
15.4	0.232854631	0.042514208	0.018448997	0.011387236
15.5	0.244593494	0.046859674	0.020508606	0.009664572
15.6	0.245440404	0.048933766	0.018863388	0.016756959
15.7	0.250382139	0.054084404	0.026080121	0.012254381
15.8	0.260379069	0.049850802	0.025689424	0.015077016
15.9	0.273280726	0.056559647	0.023645676	0.01603648
16	0.274460759	0.054672375	0.034653215	0.009141011
16.1	0.313951057	0.086548216	0.054322779	0.018654813
16.2	0.28946614	0.058738143	0.026510266	0.017755191

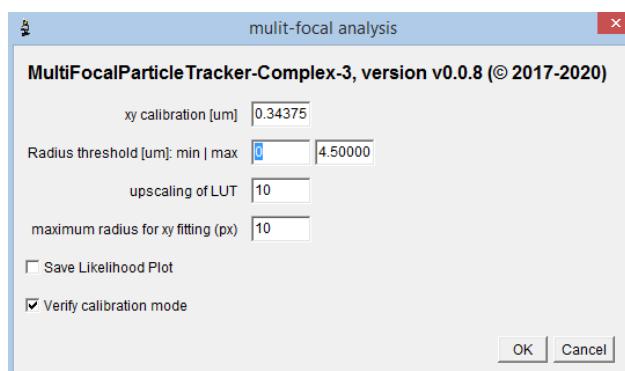
Verify the calibration Look-Up-Table (LUT)

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.

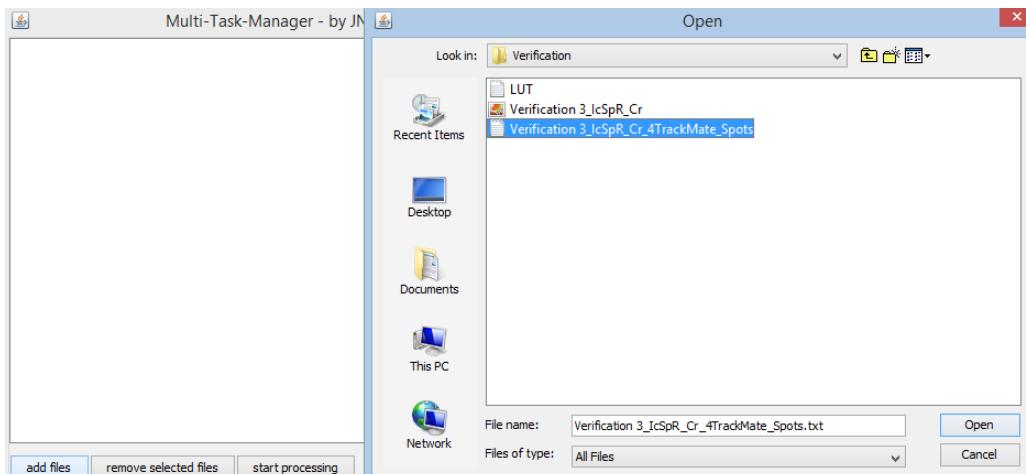
- Open ImageJ
- Install the latest release of the plugin MultiFocalParticleTracker-Complex-3: <https://github.com/hansenjn/MultiFocalParticleTracker-Complex-3/releases/>
- Restart ImageJ
- Launch MultiFocalParticleTracker-Complex-3: Plugins > JNH > Multi Focal > Complex Particle Tracking 3...
- Enter 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.

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

- 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

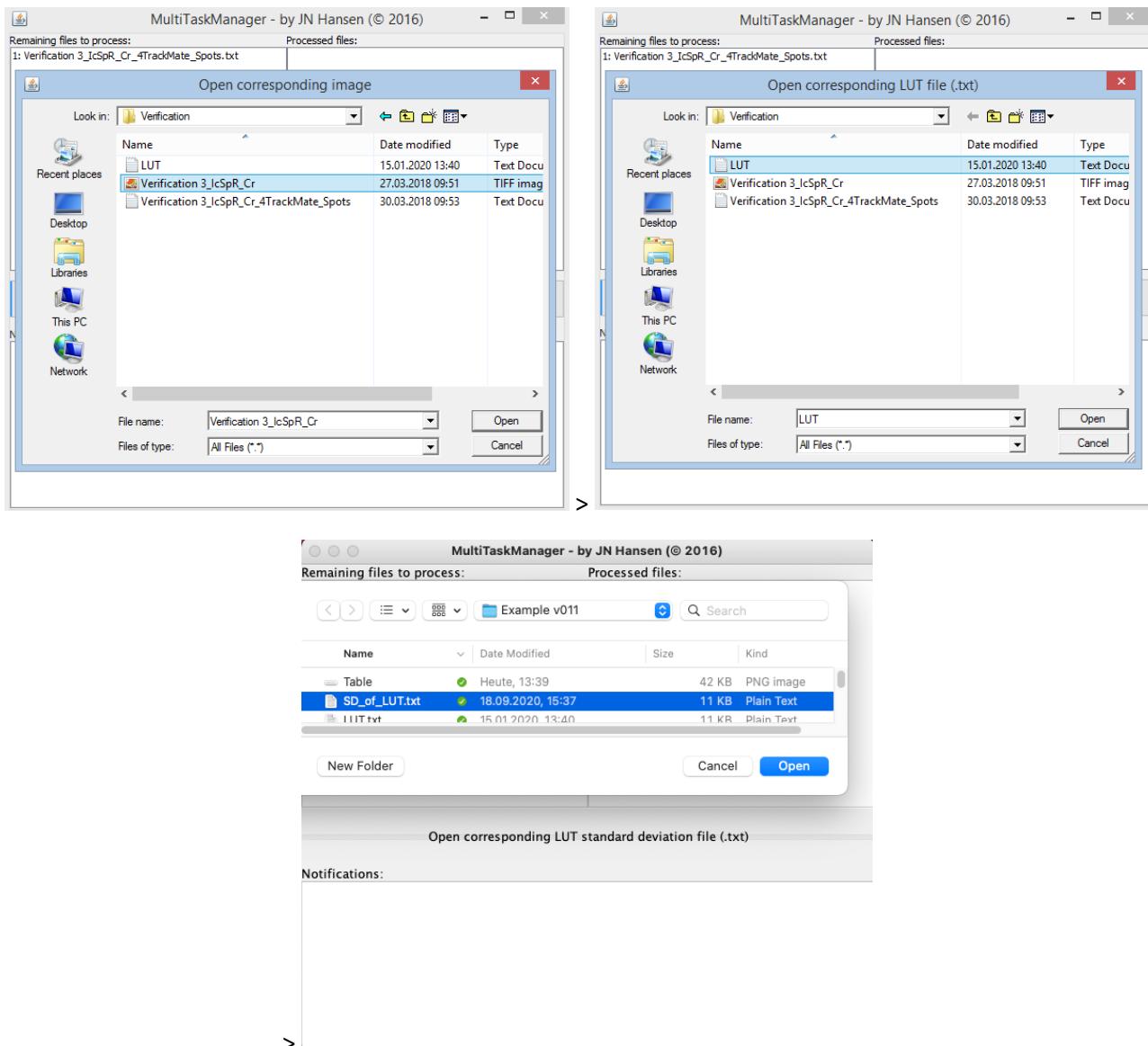


- A dialog pops up: add the text file containing the bead positions and press “start processing”:

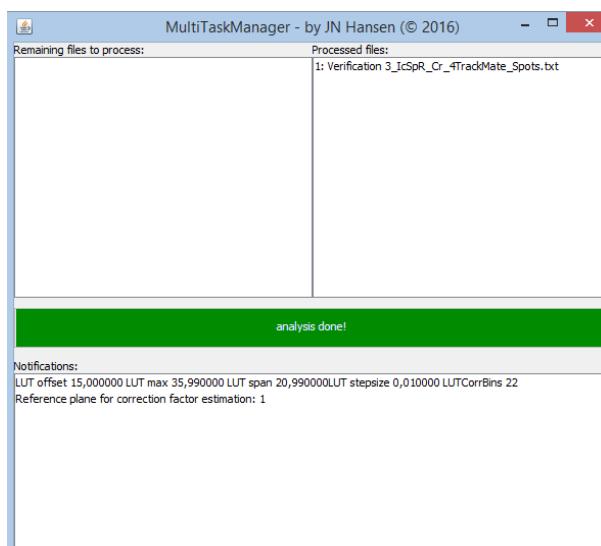


- A dialog pops up requesting to open the corresponding image. Select the corresponding image and press Open. Next, a dialog pops up requesting you to load the LUT. Select the LUT and press Open. **New from MultiFocalParticleTracker-Complex-3 version v0.1.1 on:** Next, a dialog pops up requesting to load the “LUT standard deviation file”. Select the SD_of_LUT.txt file.

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

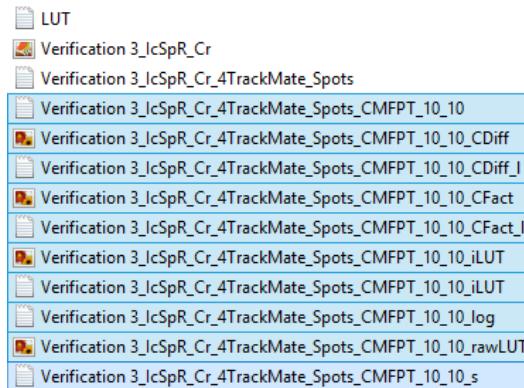


- Wait until MultiTaskManager states “analysis done!”

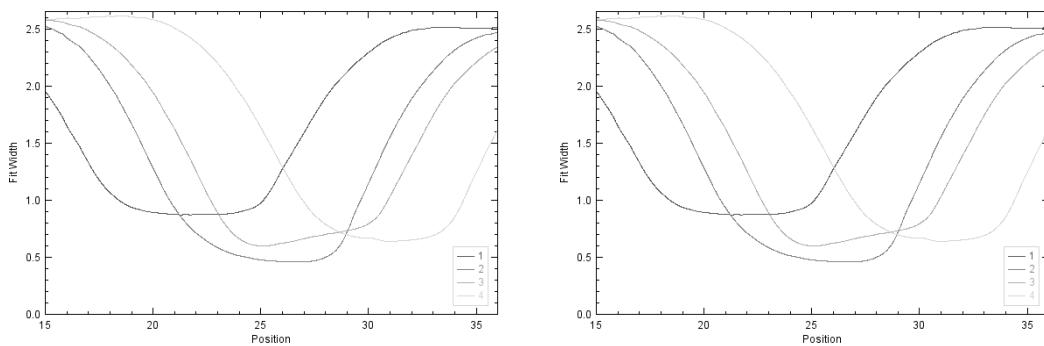


More information, license notes, credits: <https://github.com/hansenjn/MultifocalImaging-AnalysisToolbox>

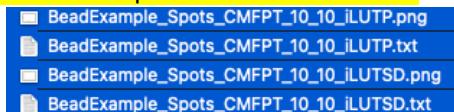
- The plugin has saved new files to the folder where the text file with bead position was saved. They all contain an additional suffix ("`_CMFPT_<numbers>_`") and specific file endings.



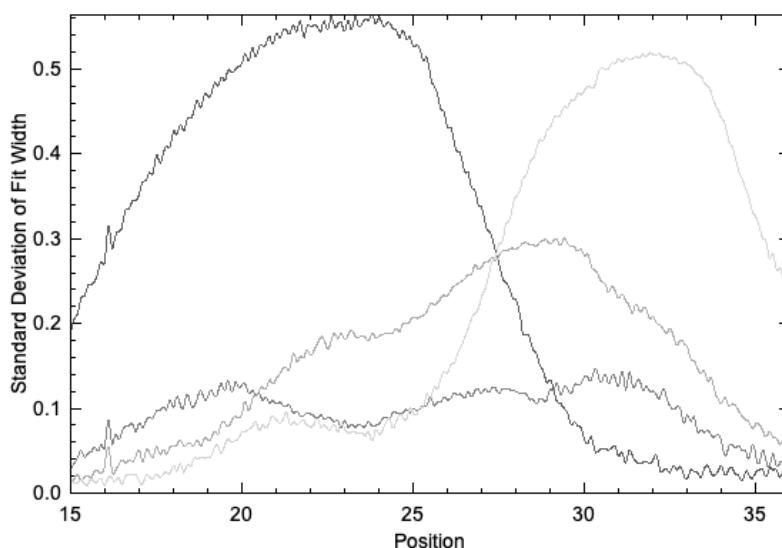
- ...rawLUT.png shows a plot of the LUT before and ...iLUT.png after spline interpolation:



- New from MultiFocalParticleTracker-Complex-3 version v0.1.1 on - additional files are output:

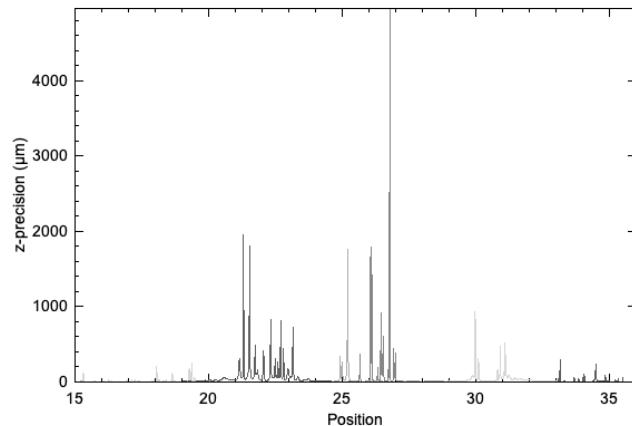


- ...iLUTSD.png shows a plot of the standard deviation of the LUT after spline interpolation (for each plane, a different "gray" is used for plotting):



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

- ...iLUTP.png shows a plot of the predicted precision for inferring the z-position from the bead width at the specific z-position (for each plane, a different “gray” is used for plotting):



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

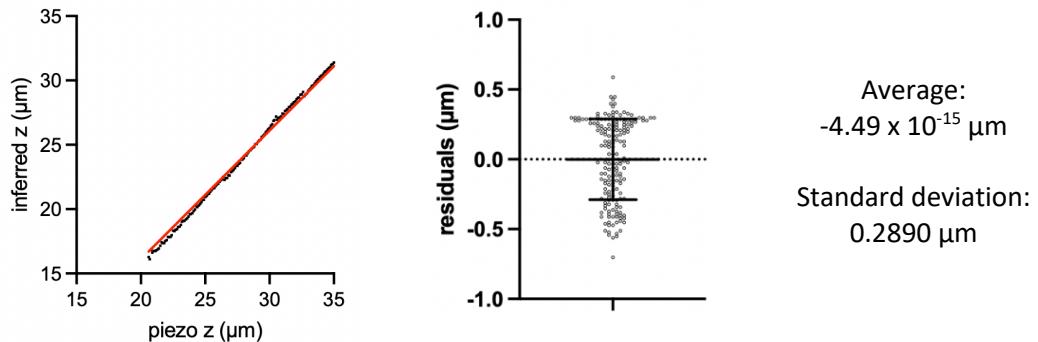
Verification 3_lcSpR_Cr_4TrackMate_Spots_CMFPT_10_10_s - Notepad						30.05.2020 23:18	Text Document
File	Edit	Format	View	Help			
index	T	X (μm)	Y (μm)	Z average (μm)	factor-corr.	Z avg (μm)	^
0	0,000000	75,678969		58,700125		35,093333	
1	1,000000	75,678969		58,700125		34,645000	
2	2,000000	75,678969		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	

- Changed from MultiFocalParticleTracker-Complex-3 version v0.1.1 on: Extract the columns 2 to 4 (they show the slice position (T) and the X and Y coordinate of the bead) and column 8 (shows the baeds z-position, inferred from the plane yielding the best predicted precision).

	A	B	C	D	E	F	G	H	I	J	K	L
1	index	T	X (μm)	Y (μm)	Z average (μm)	factor-corr.	Z from precisiest	radius plane 1	radius plane 2	radius plane 3	radius plane 4	ch
2	0.00	0.00	13.88	13.67	21.97	22.19	22.22	22.57	0.34	0.76	1.14	2.28 21.
3	1.00	1.00	13.81	13.63	21.93	22.16	22.18	22.42	0.34	0.76	1.15	2.31 21.
4	2.00	2.00	13.69	13.68	21.99	22.21	22.24	22.57	0.34	0.74	1.15	2.28 21.

- 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 scripts 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:



Bead tracking in 3D

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”).

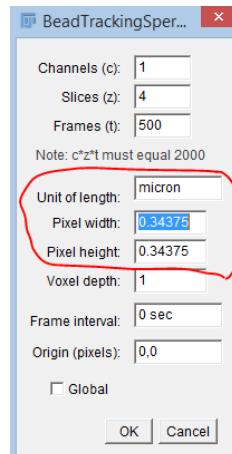
Next, generate a text file containing the bead positions over time using TrackMate (<https://imagej.net/TrackMate>) as explained in the following. It is recommended to not install TrackMate manually into your ImageJ but rather download and use the FIJI distribution of ImageJ, where the plugin TrackMate 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

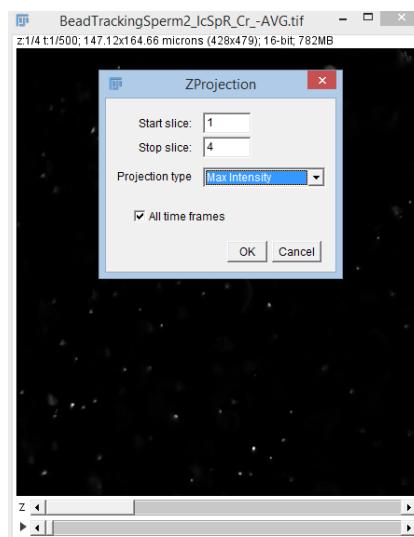


- Make sure the image is correctly calibrated: Image > Properties; If the information provided at “Unit of length”, “Pixel width”, and “Pixel height” is incorrect, correct it; Press OK.

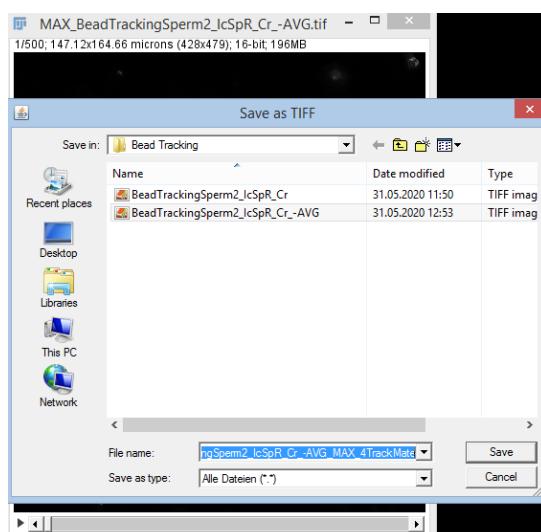
More information, license notes, credits: <https://github.com/hansenjn/MultifocalImaging-AnalysisToolbox>



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



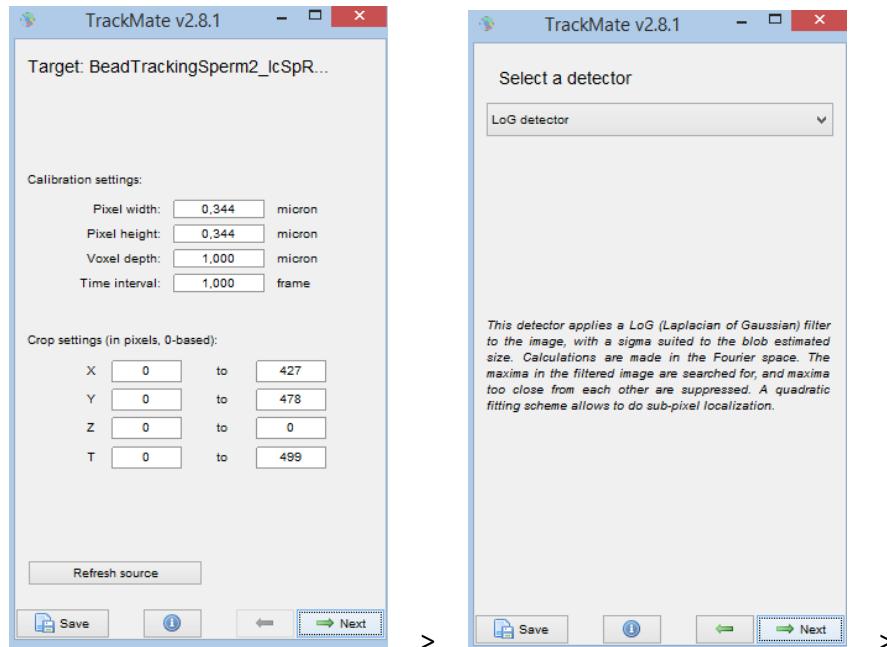
- Save the stack with the ending “_MAX_4TrackMate.tif” to your directory: File > Save As > Tiff...



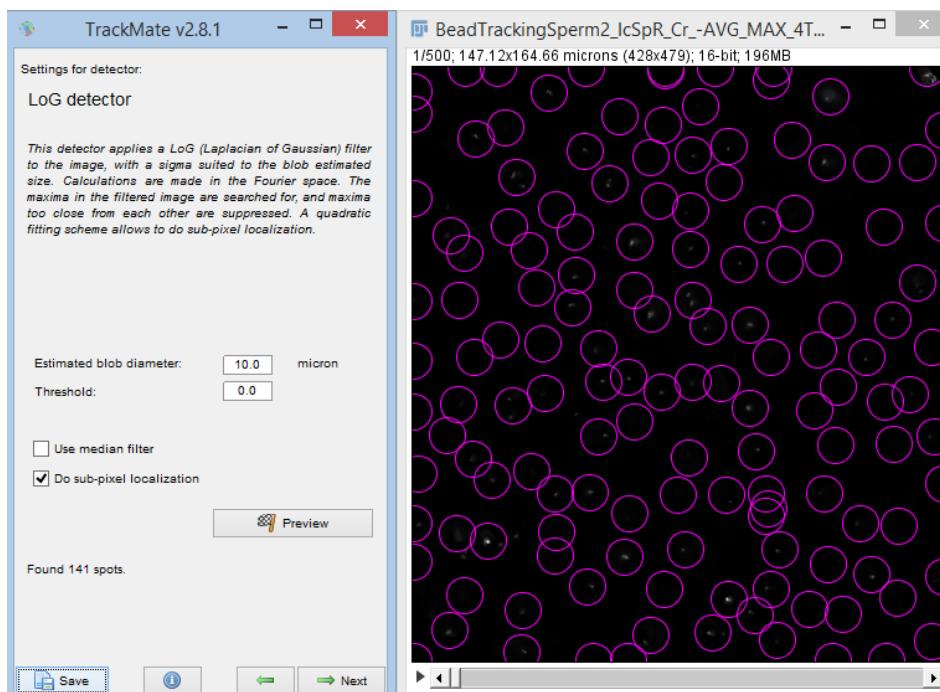
- Launch TrackMate: Plugins > Tracking > TrackMate; if a dialog pops up asking you to swap Z and T confirm by pressing Yes (or “Ja” if you have a German computer):



- A dialog pops up - press Next:



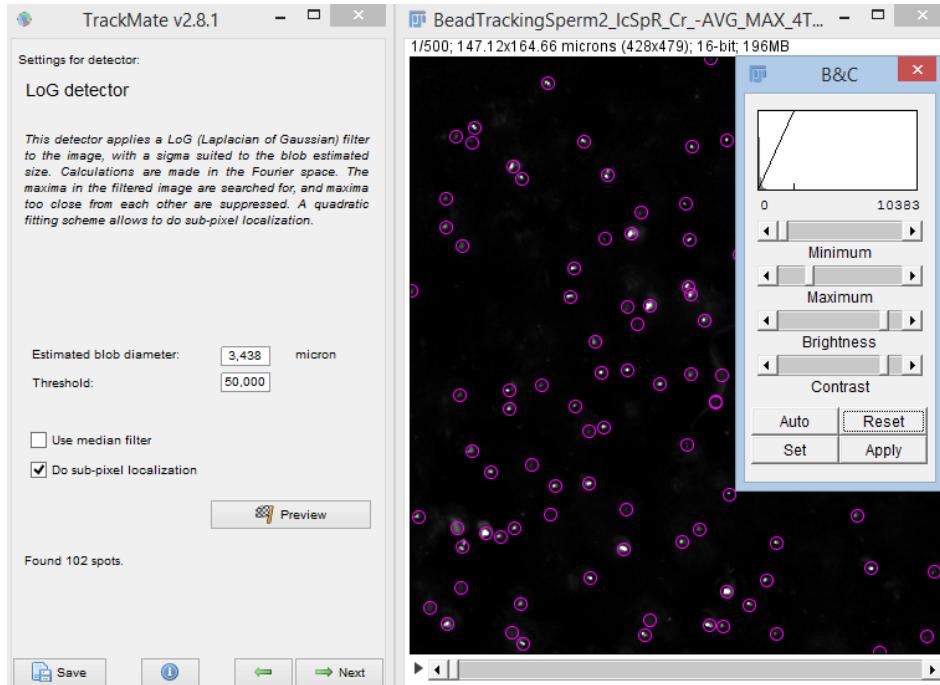
- Press Next and then "Preview" to see the positions that will be detected as a particle (will be encircled in purple)



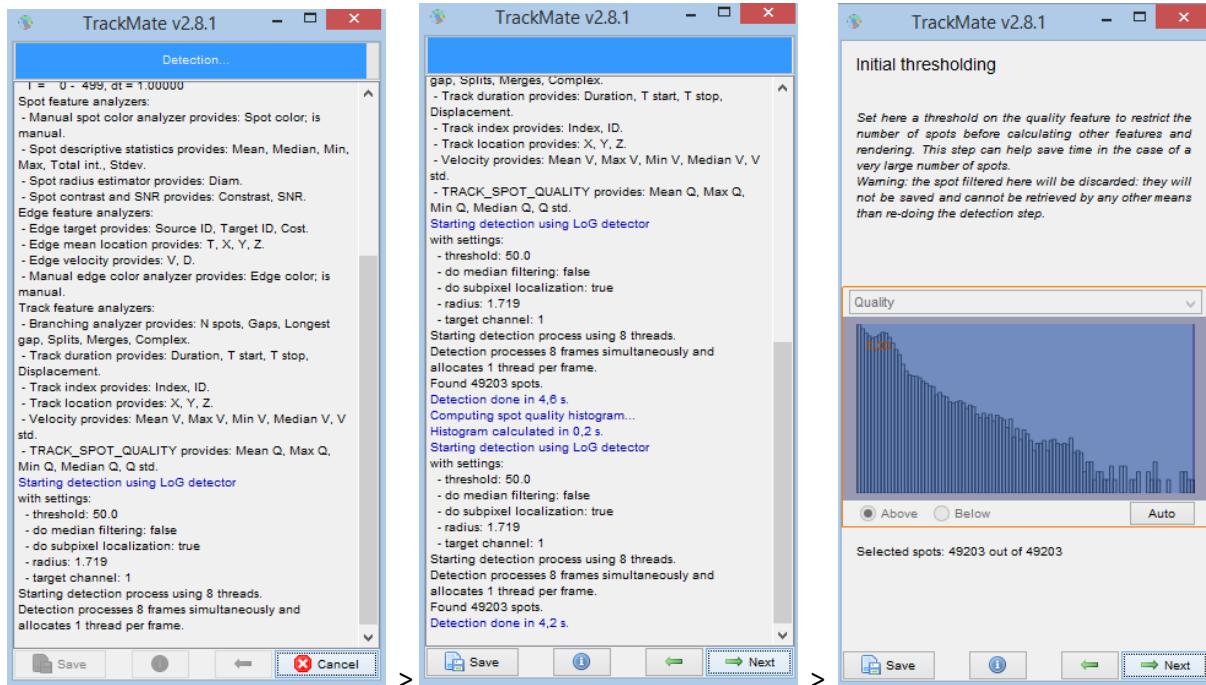
- Adapt the blob diameter and the threshold until the detection is correct (press preview to preview whether settings have improved detection). Eventually, you might need to

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

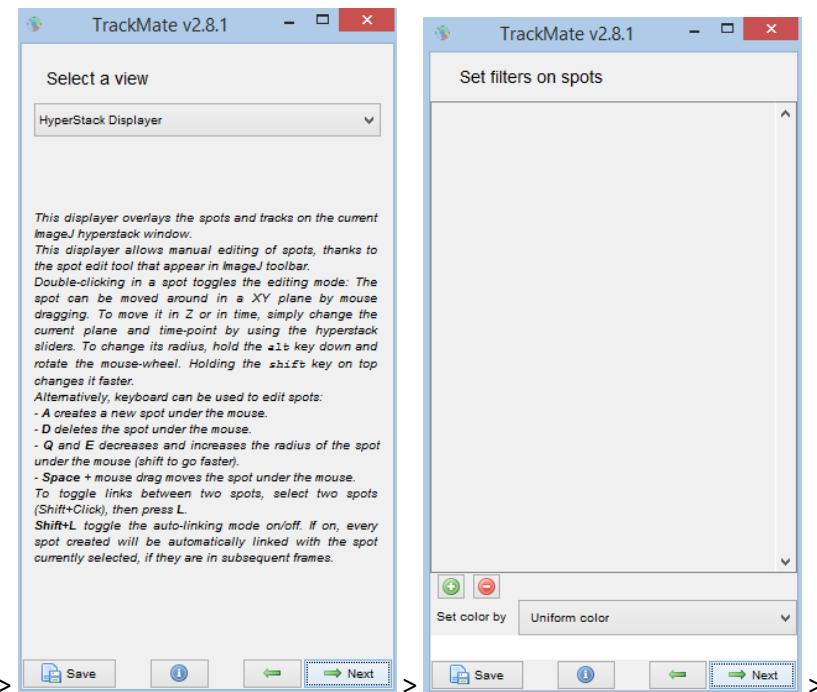
increase the Brightness / Contrast (Image > Adjust > Brightness / Contrast) to better see where beads are located.



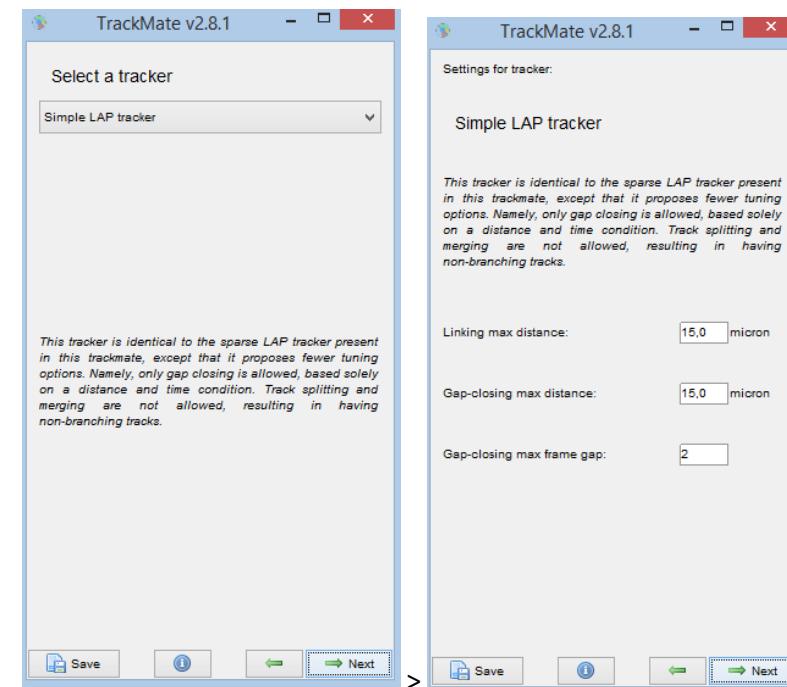
- Click five times Next until you reach a dialog showing “Select Tracker”:



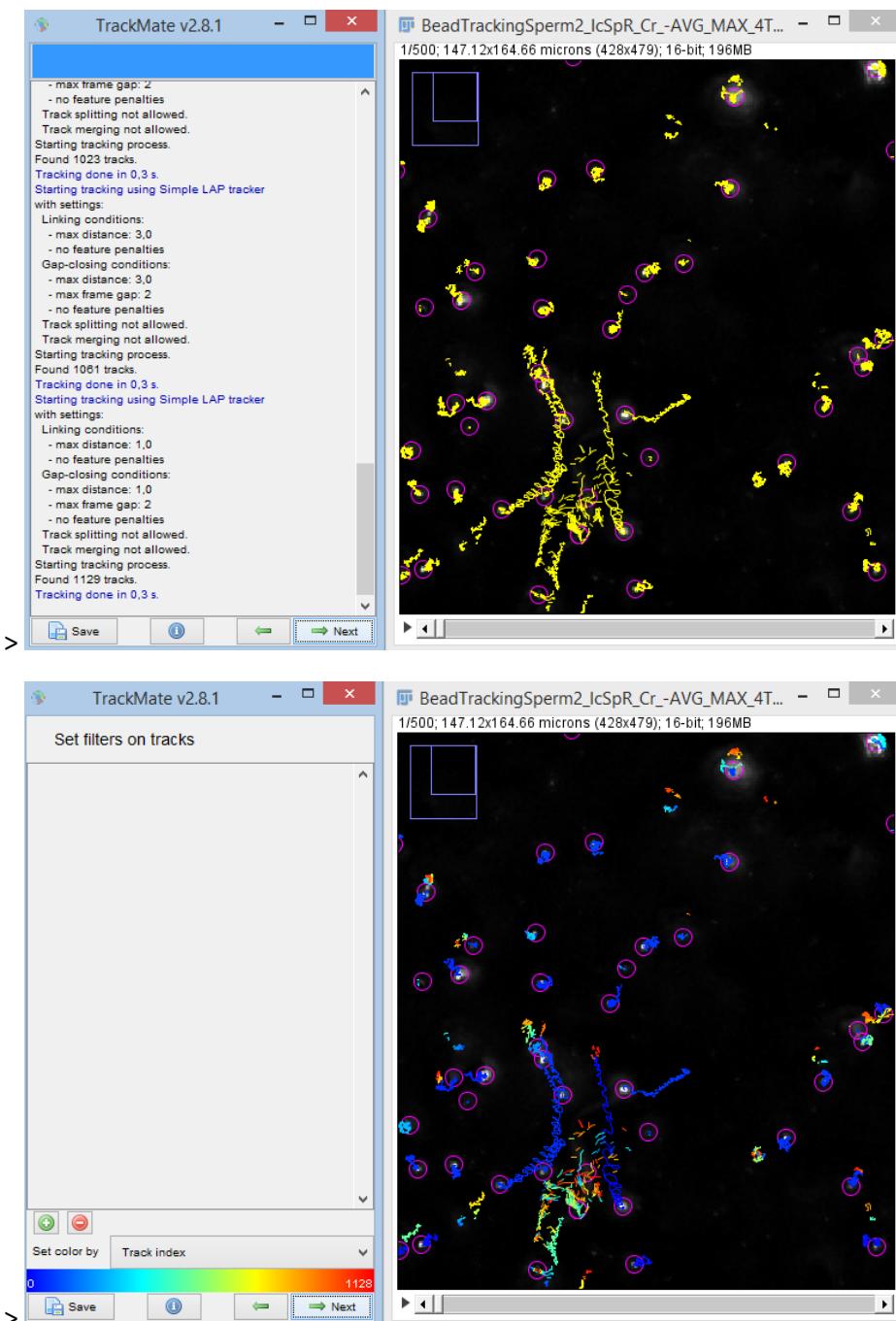
More information, license notes, credits: <https://github.com/hansenjn/MultifocalImaging-AnalysisToolbox>



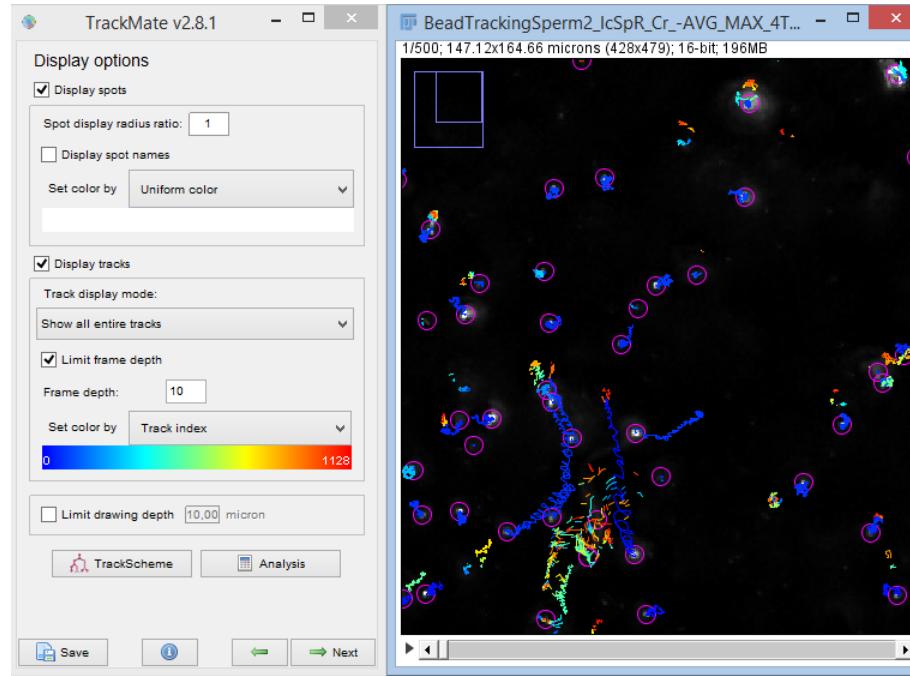
- In the dialog “Select a tracker”, select “Nearest neighbor search” and press Next for four times:



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



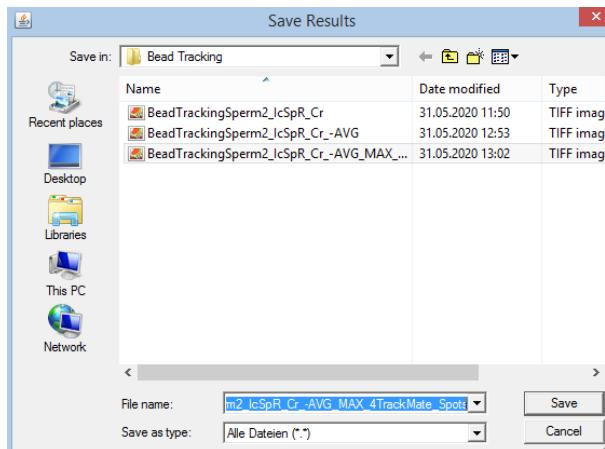
More information, license notes, credits: <https://github.com/hansenjn/MultifocalImaging-AnalysisToolbox>



- 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:

Track statistics				Links in tracks statistics				Spots in tracks statistics								
File	Edit	Font	Label	NUMBER_SPOTS	NUMBER_GAPS	LONGEST_TRACK	Label	TRACK_ID	SPOT_SOURCE_ID	Label	ID	TRACK_ID	QUALITY	POSITION		
1	Track_2897	326	0	0			1	(ID49827 : ID49924)	0	49827	1	ID49827	49827	0	1053.79675	87.24324
2	Track_2898	376	1	1			2	(ID49924 : ID49629)	0	49924	2	ID49924	49924	0	1007.56110	87.29112
3	Track_2899	500	0	0			3	(ID49629 : ID50020)	0	49629	3	ID49629	49629	0	1136.19006	87.31785
4	Track_2900	500	0	0			4	(ID50020 : ID50116)	0	50020	4	ID50020	50020	0	999.36249	87.28498
5	Track_2901	12	0	0			5	(ID50116 : ID49726)	0	50116	5	ID50116	50116	0	1340.79919	87.33927
6	Track_2902	500	0	0			6	(ID49726 : ID49533)	0	49726	6	ID49726	49726	0	1342.32104	87.33234
7	Track_2903	500	0	0			7	(ID49533 : ID50215)	0	49533	7	ID49533	49533	0	1336.68665	87.31784
8	Track_2904	500	0	0			8	(ID50215 : ID50311)	0	50215	8	ID50215	50215	0	1403.55603	87.35430
9	Track_2905	3	0	0			9	(ID50311 : ID50602)	0	50311	9	ID50311	50311	0	1300.04761	87.34724
10	Track_2906	366	0	0			10	(ID50602 : ID50475)	0	50602	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 ...

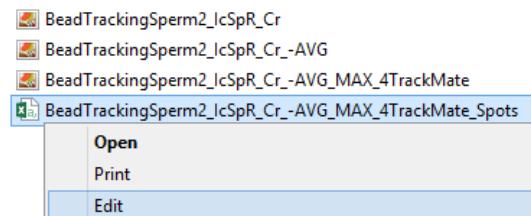


- Close all images and windows in Fiji.

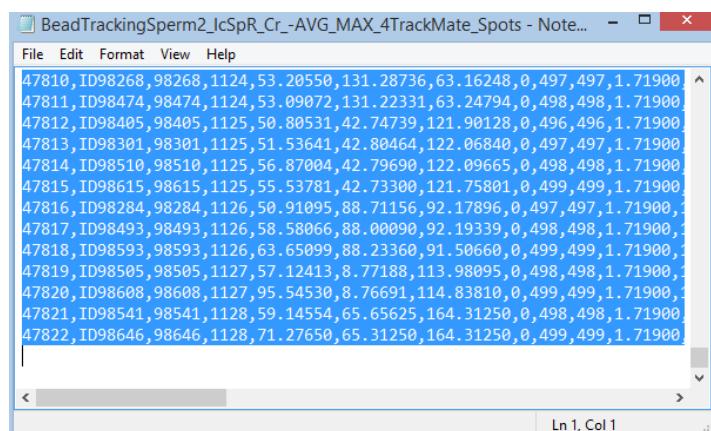
More information, license notes, credits: <https://github.com/hansenjn/MultifocalImaging-AnalysisToolbox>

- 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:

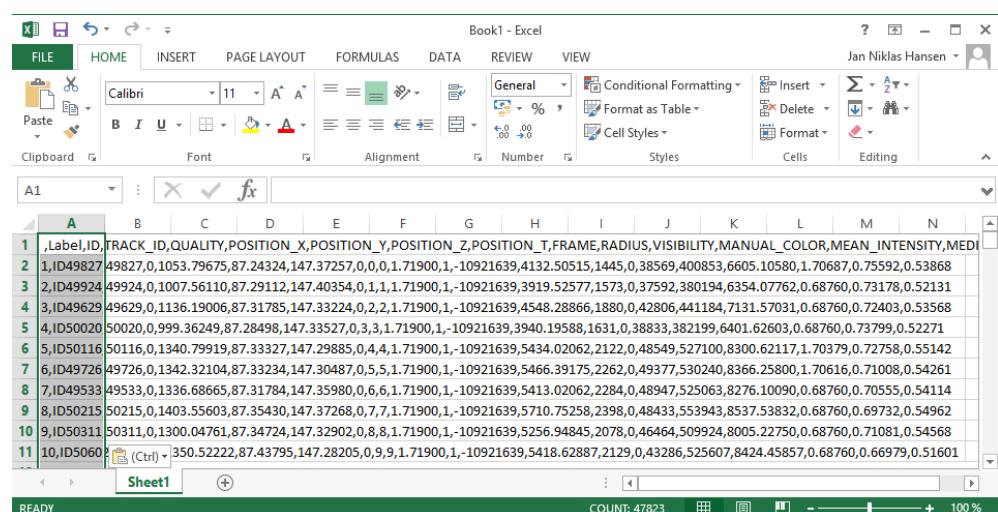
- Open the saved .csv file with a text editor (right-click on file > Edit)



- Mark all and press Ctrl + C:



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

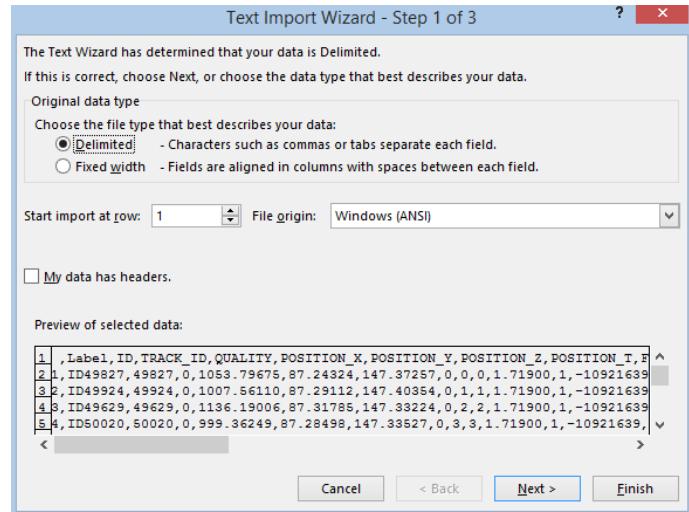


- Click on the small Ctrl button on the bottom and select “Use Text Import Wizard...”

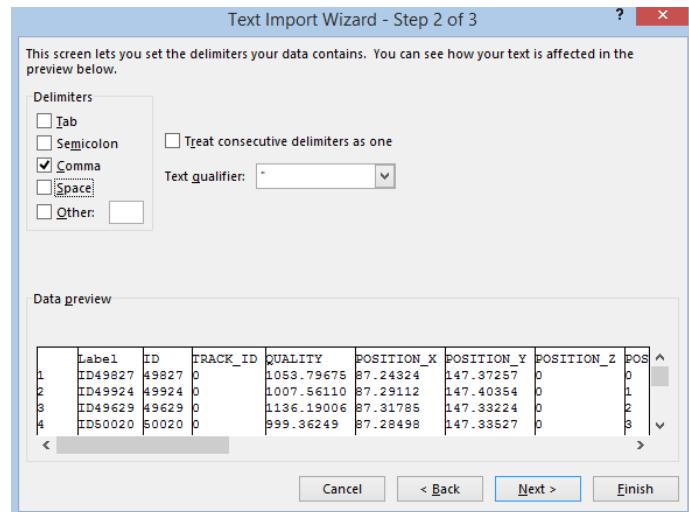
More information, license notes, credits: <https://github.com/hansenjn/MultifocalImaging-AnalysisToolbox>

1	,Label, ID, TRACK_ID, QUALITY, POSITION_X, POSITION_Y, POSITION_Z, POSITION_T, F
2	1, ID49827, 49827, 0, 1053.79675, 87.24324, 147.37257, 0, 0, 0, 1.71900, 1, -10921639
3	2, ID49924, 49924, 0, 1007.56110, 87.29112, 147.40354, 0, 1, 1, 1.71900, 1, -10921639
4	3, ID49629, 49629, 0, 1136.19006, 87.31785, 147.33224, 0, 2, 2, 1.71900, 1, -10921639
5	4, ID50020, 50020, 0, 999.36249, 87.28498, 147.33527, 0, 3, 3, 1.71900, 1, -10921639, v

- 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. 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 data structure: POSITION_T, POSITION_X, and POSITION_Y.

	A	B	C	D	E	F	G	H	I	J
1	Label	ID	TRACK_I	QUALITY	POSITION_X	POSITION_Y	POSITION_T	FRAME	PAE	
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	ID50898	50898	0	1514.7	87.47344	147.19875	0	14	14
17	16	ID50947	50947	0	1514.6	87.47649	147.19489	0	15	15

	A	B	C
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	87.47344	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 the Bead Tracking software interface. The main window displays a table of bead positions with columns POSITION_T, POSITION_X, and POSITION_Y. The Notepad window shows the raw data from the text file, listing each position with its corresponding timestamp, x-position, and y-position.

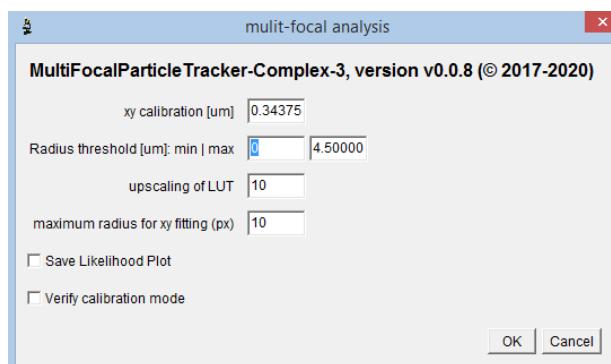
	A	B	C
1	POSITION_T	POSITION_X	POSITION_Y
2	0	87.24324	147.37257
3	1	87.29112	147.40354
4	2	87.31785	147.33224
5	3	87.28498	147.33527
6	4	87.33327	147.29885
7	5	87.33234	147.30487
8	6	87.31784	147.35938
9	7	87.3543	147.37268
10	8	87.34724	147.32902
11	9	87.43795	147.28205
12	10	87.3945	147.28699
13	11	87.47731	147.31607
14	12	87.53394	147.35636
15	13	87.54117	147.24707
16	14	87.47344	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.

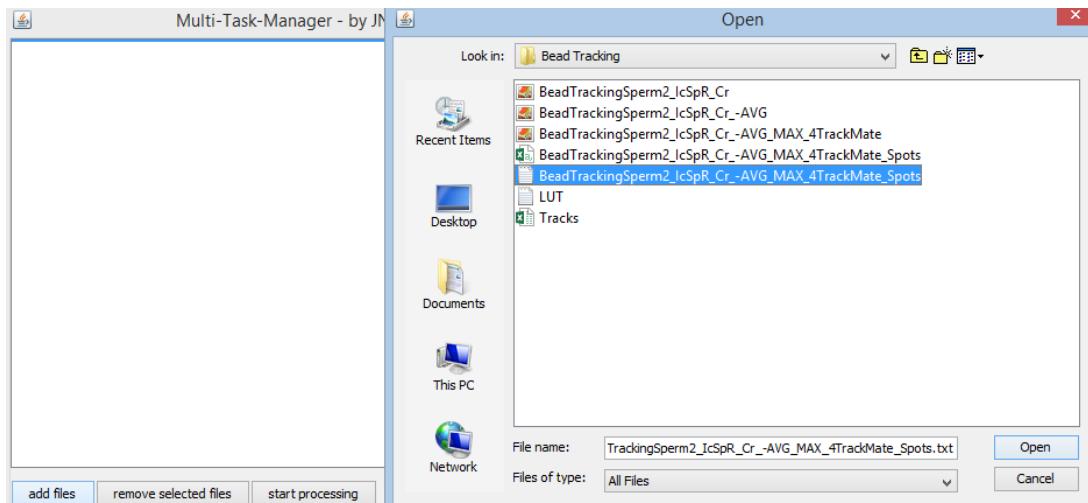
- Open ImageJ
- If not already installed (this should have been installed for verifying the LUT), install the latest release of the plugin MultiFocalParticleTracker-Complex-3 (<https://github.com/hansenjn/MultiFocalParticleTracker-Complex-3/releases/>) and restart ImageJ
- Launch MultiFocalParticleTracker-Complex-3: 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

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

- 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”:

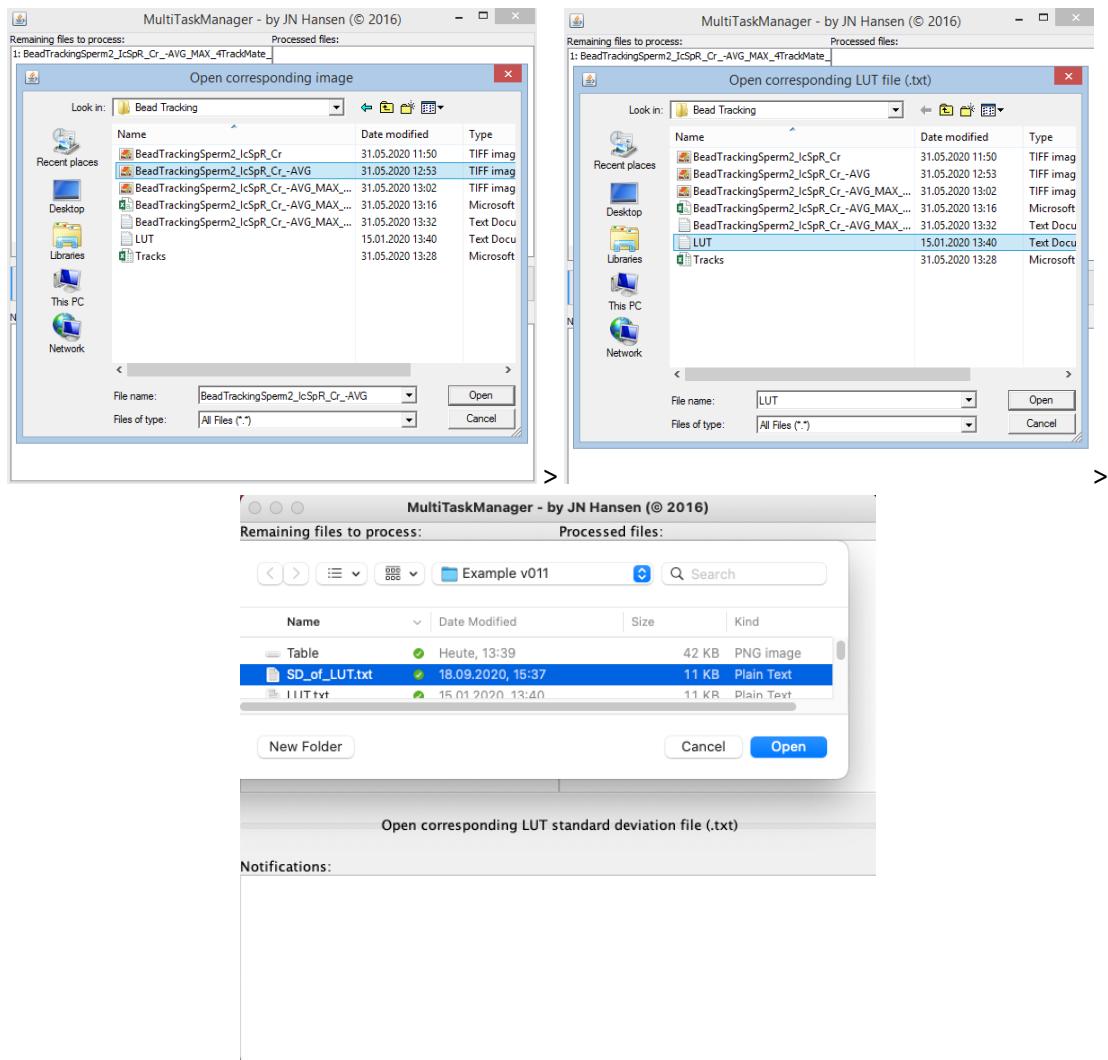


- A dialog pops up requesting to open the corresponding image. Select the corresponding image and press Open. Next, dialog pops up requesting you to load the LUT. Select the LUT and press Open. **New from MultiFocalParticleTracker-Complex-3 version v0.1.1 on:** Next, a dialog pops up requesting to load the “LUT standard deviation file”. Select the SD_of_LUT.txt file.

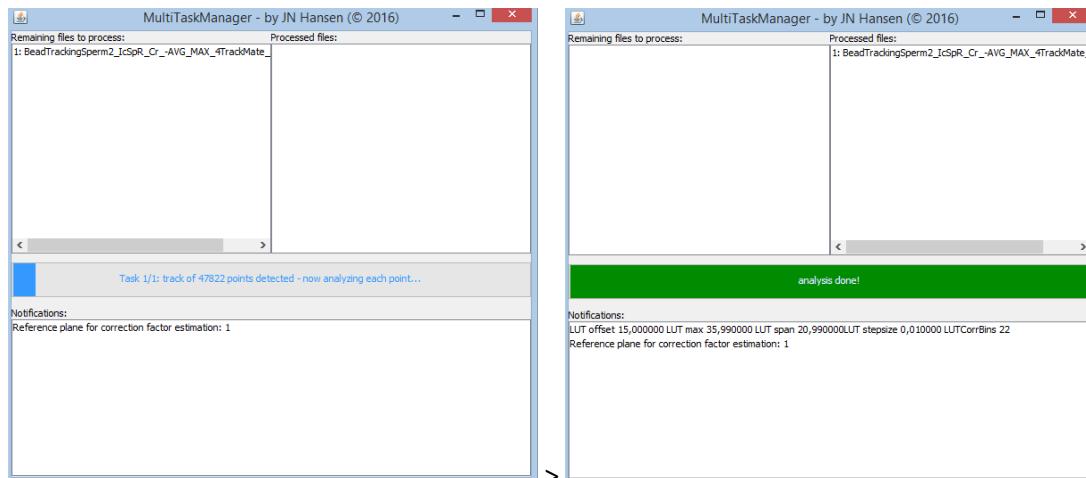
>

-

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

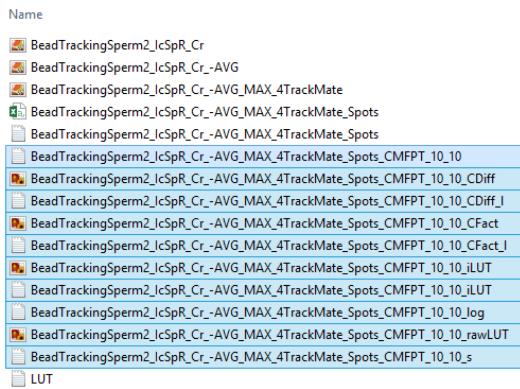


- Wait until MultiTaskManager states “analysis done!”

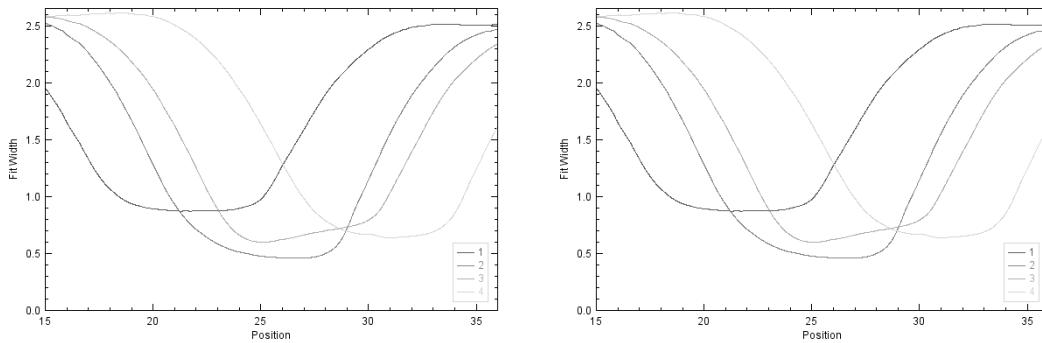


- The plugin has saved new files to the folder where the text file with bead position was saved. They all contain an additional suffix (“_CMFPT_<numbers>_”) and specific file endings.

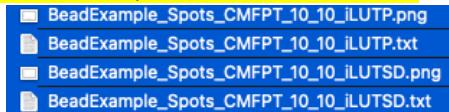
More information, license notes, credits: <https://github.com/hansenjn/MultifocalImaging-AnalysisToolbox>



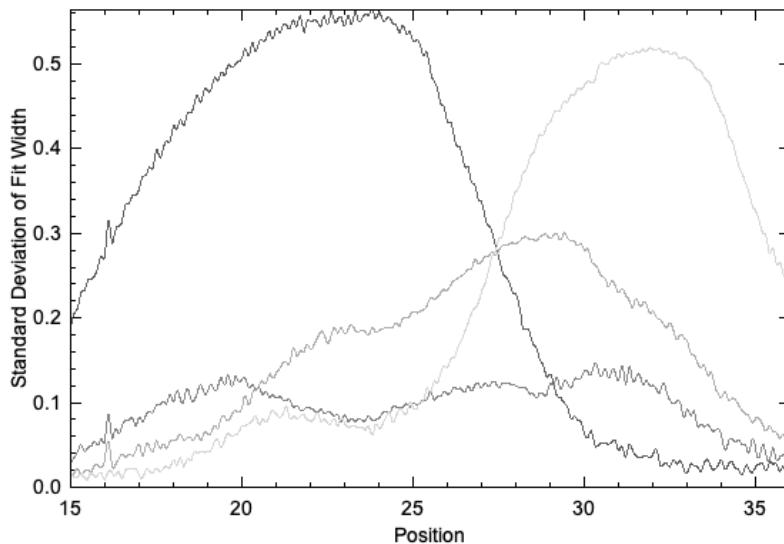
- ...rawLUT.png shows a plot of the LUT before and ...iLUT.png after spline interpolation:



- New from MultiFocalParticleTracker-Complex-3 version v0.1.1 on - additional files are output:

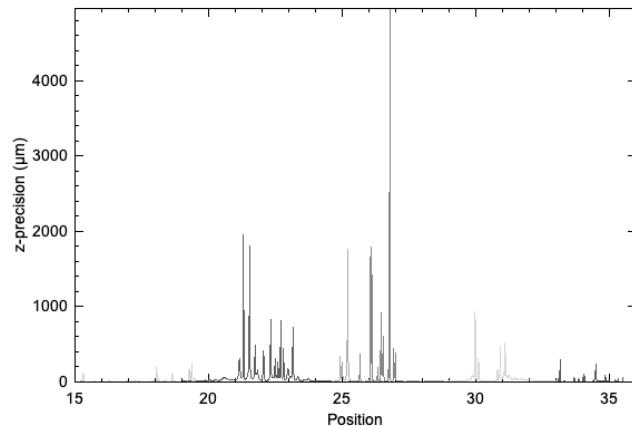


- ...iLUTSD.png shows a plot of the standard deviation of the LUT after spline interpolation (for each plane, a different “gray” is used for plotting):



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- ...iLUTP.png shows a plot of the predicted precision for inferring the z-position from the bead width at the specific z-position (for each plane, a different “gray” is used for plotting):



- 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							
File	Edit	Format	View	Help			
index	T	X (μm)	Y (μm)	Z average (μm)	factor-corr.	Z avg (μm)	
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	

- Changed from MultiFocalParticleTracker-Complex-3 version v0.1.1 on: Extract the columns 2 to 4 (they show the slice position (T) and the X and Y coordinate of the bead) and column 8 (shows the baeds z-position, inferred from the plane yielding the best predicted precision).

	A	B	C	D	E	F	G	H	I	J	K	L
1	index	T	X (μm)	Y (μm)	Z average (μm)	factor-corr.	diff-corr.	Z from precisiest plane (μm)	radius plane 1 (μm)	radius plane 2 (μm)	radius plane 3 (μm)	radius plane 4 (μm)
2	0.00	0.00	13.88	13.67	21.97	22.19	22.22	22.57	0.34	0.76	1.14	2.28
3	1.00	1.00	13.81	13.63	21.93	22.16	22.18	22.42	0.34	0.76	1.15	2.31
4	2.00	2.00	13.69	13.68	21.99	22.21	22.24	22.57	0.34	0.74	1.15	2.28
5	3.00	3.00	13.67	13.70	22.04	22.26	22.20	22.52	0.33	0.72	1.14	2.25

- 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:

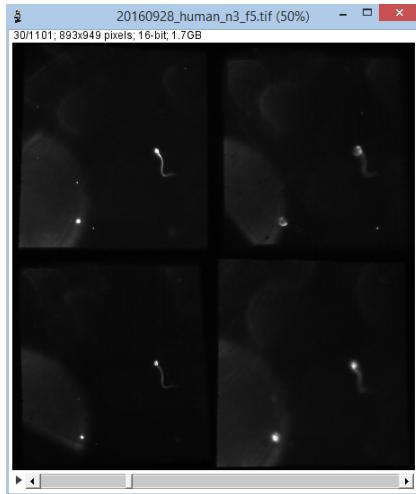
<https://github.com/hansenjn/MultifocalImaging-AnalysisToolbox/tree/master/Matlab%20scripts>

The MATLAB script additionally implements connecting the bead positions to tracks, filtering the tracks, plotting the tracks, and more...

3D reconstruction of flagella

Requirements

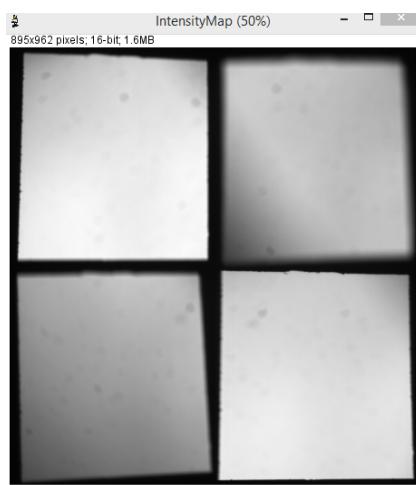
1. Multifocal imaging recording of beating flagella



2. A multifocal imaging piezo-generated z stack through non-moving flagella, allowing to obtain a calibration between flagellar width and z position

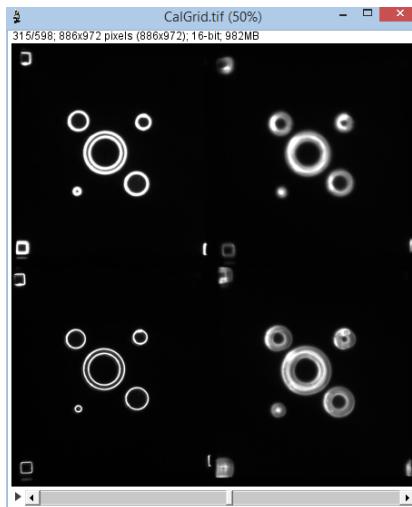


3. A reference file for intensity corrections (obtained by imaging without a specimen)



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

4. A reference file for aligning the planes and determining focal positions: record a piezo driven z-stack through a calibration grid. If calibration grid not available, record beads or other specimens that are thin and localize mainly to one focal plane – it is important that you record an image of (a) structure(s) that spans the field of view in all directions; if this is not the case, e.g. when using an image showing only one flagellum, alignment can be incorrect, because in the blank image regions (e.g. perpendicular to the flagellum), structures are missing that allow the alignment).



Workflow

Creating an intensity map

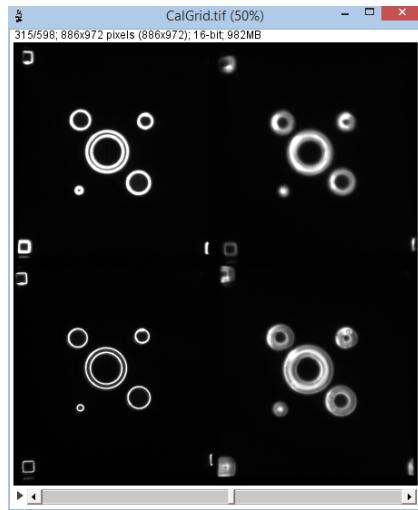
An intensity map is created out of the reference file for intensity corrections as described for bead tracking (see above).

Creating a registration file & determining focal positions

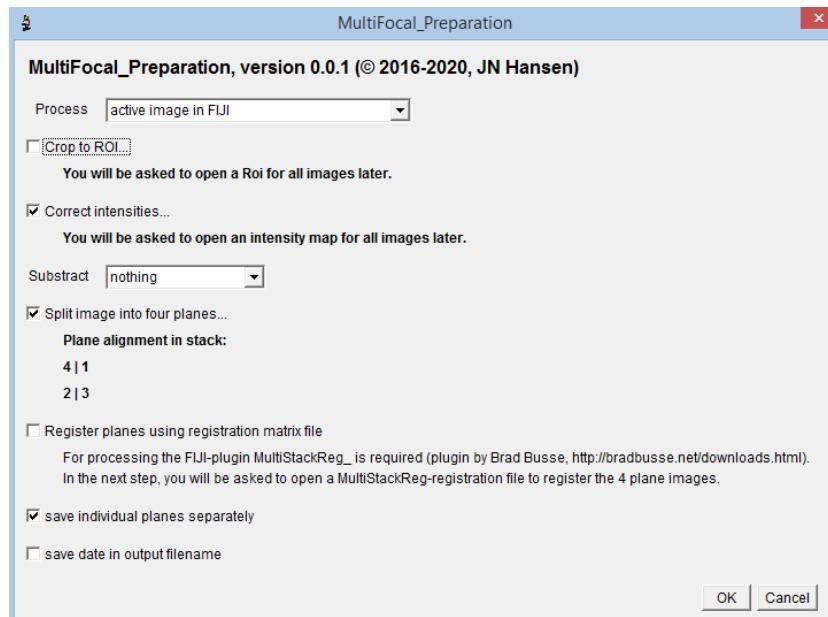
Use the piezo-driven z-stack of a calibration grid (called “reference file for aligning the planes and determining focal positions” above) to determine focal positions and obtain a registration file as follows.

- If not already installed:
 - Install the plugin MultiStackReg from Brad Busse: <http://bradbisse.net/downloads.html>
 - Install the latest release from the plugin Multifocal_Preparation:
https://github.com/hansenjn/MultiFocal_Preparation/releases
 - Restart ImageJ
- Open the image stack of the calibration grid

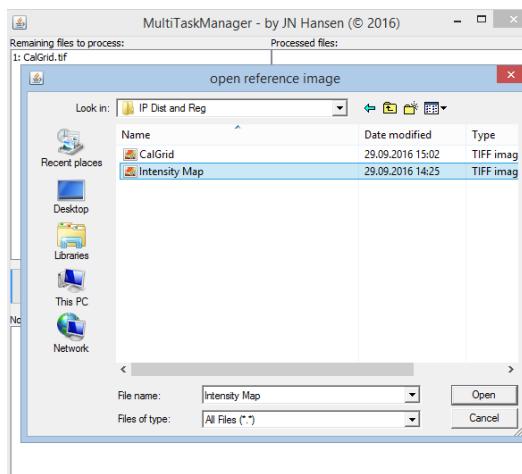
More information, license notes, credits: <https://github.com/hansenjn/MultifocalImaging-AnalysisToolbox>



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

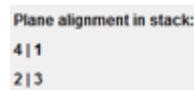


- In the upcoming dialog, select the intensity map produced as described in the previous section as a reference image (in the image below: "Intensity Map.tif") and press "Open":

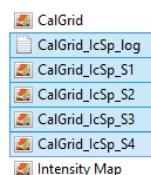


More information, license notes, credits: <https://github.com/hansenjn/MultifocalImaging-AnalysisToolbox>

- 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 image stack and is output
 - 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:

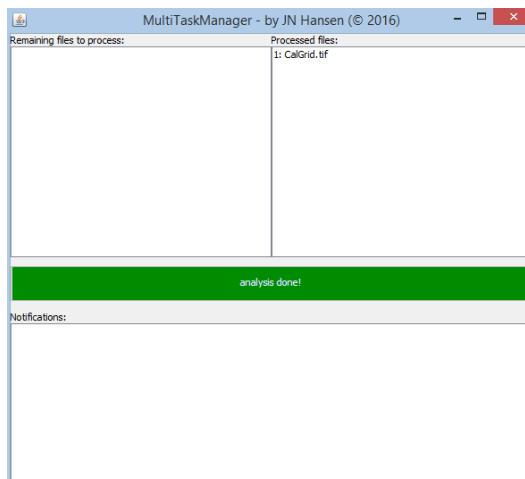


- At the end of the processing, MultiFocal_Preparation saves new files to the repository where the input file was saved:

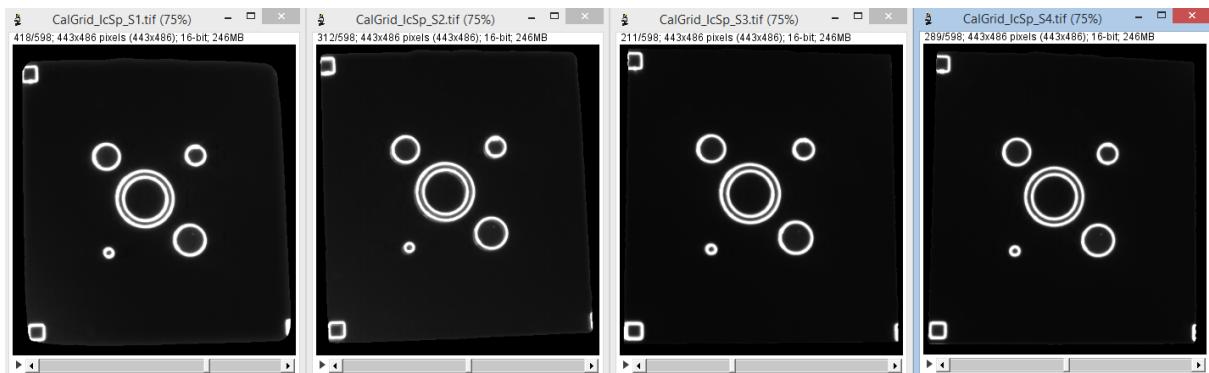


- The .tif-files with ending lcSp_S1, lcSp_S2, etc. contain the stacks for each individual plane (“1”, “2”, “3”, “4”)
- The text file documents the processing settings of “MultiFocal_Preparation”.

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

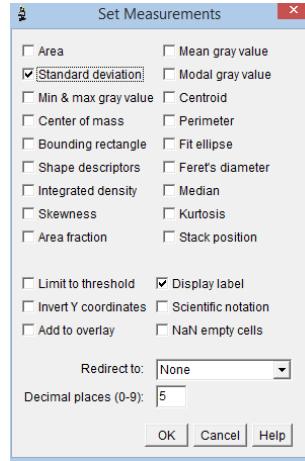


- Open all four "..._lcSp_S<1,2,3,4>.tif" files in ImageJ

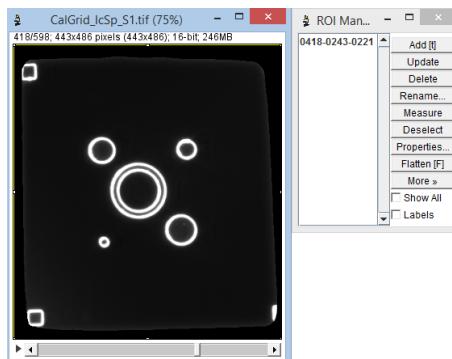


More information, license notes, credits: <https://github.com/hansenjn/MultifocalImaging-AnalysisToolbox>

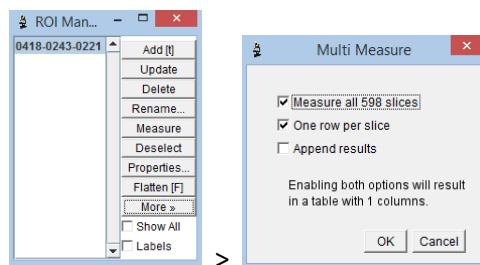
- Adapt the Measurement tool to determine the standard deviation in the image: Analyze > Set Measurements..., check the box “standard deviation”, press OK



- Set a rectangular ROI circumscribing the entire image and transfer the selection to the ROI manager: select the image stack of the first plane, press a on the keyboard, and press t on the keyboard



- Measure the standard deviation in that image: click on the selection in the ROI Manager, launch in the ROI Manager More >> Multi Measure..., select in the emerging dialog the options “Measure all ... slices” and “One row per slice”, and press OK.

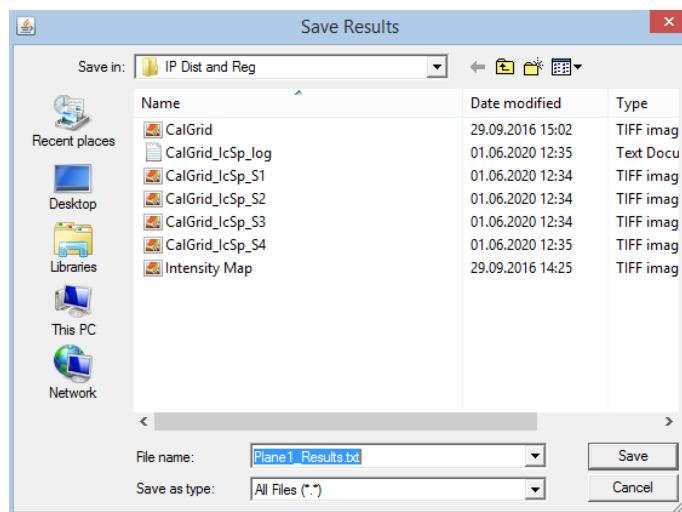


- A table pops up showing the standard deviation values of each image in the stack:

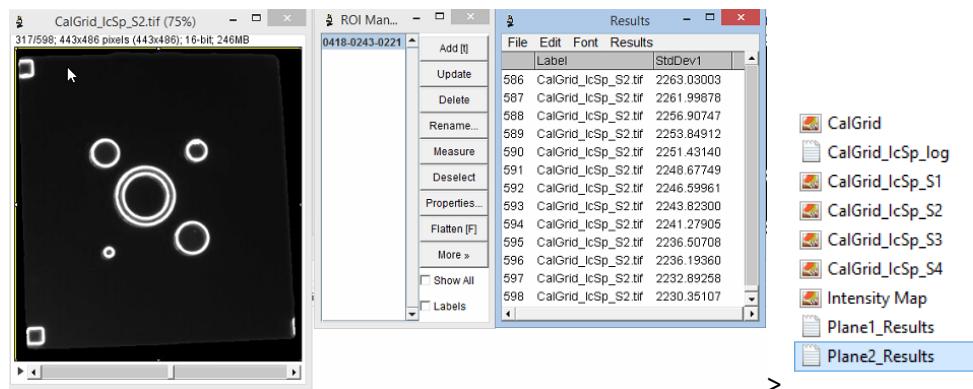
More information, license notes, credits: <https://github.com/hansenjn/MultifocalImaging-AnalysisToolbox>

	Label	StdDev1
1	CalGrid_IcSp_S1.tif	2119.76172
2	CalGrid_IcSp_S1.tif	2123.26660
3	CalGrid_IcSp_S1.tif	2124.45312
4	CalGrid_IcSp_S1.tif	2126.62817
5	CalGrid_IcSp_S1.tif	2127.79346
6	CalGrid_IcSp_S1.tif	2129.40137
7	CalGrid_IcSp_S1.tif	2132.37646
8	CalGrid_IcSp_S1.tif	2133.32104
9	CalGrid_IcSp_S1.tif	2135.78633
10	CalGrid_IcSp_S1.tif	2137.23315
11	CalGrid_IcSp_S1.tif	2139.11865
12	CalGrid_IcSp_S1.tif	2140.78955
13	CalGrid_IcSp_S1.tif	2142.13232

- Save the results as a text file to the directory (e.g. under the name “Plane1_Results.txt”): File > Save As...

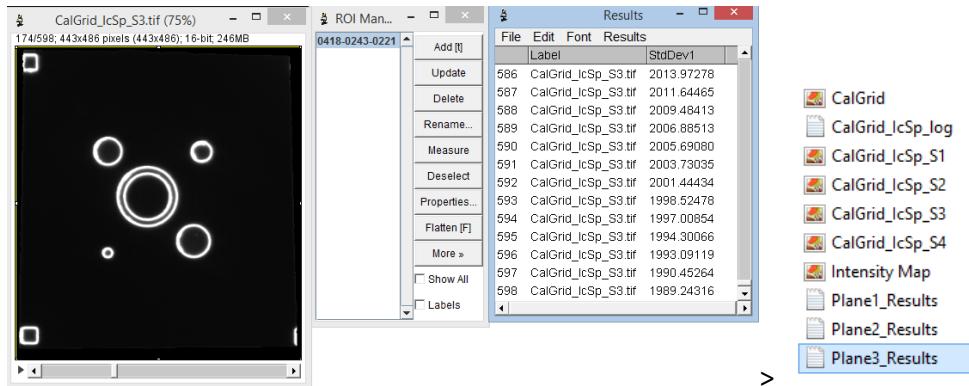


- Repeat the same analysis for the other 3 plane images
 - Click on the image of plane 2, click on the selection in the ROI Manager, launch in the ROI Manager More >> Multi Measure..., select in the emerging dialog the options “Measure all ... slices” and “One row per slice”, and press OK, Save the emerging results window as a text file to the directory (e.g. under the name “Plane2_Results.txt”) viaFile > Save As...

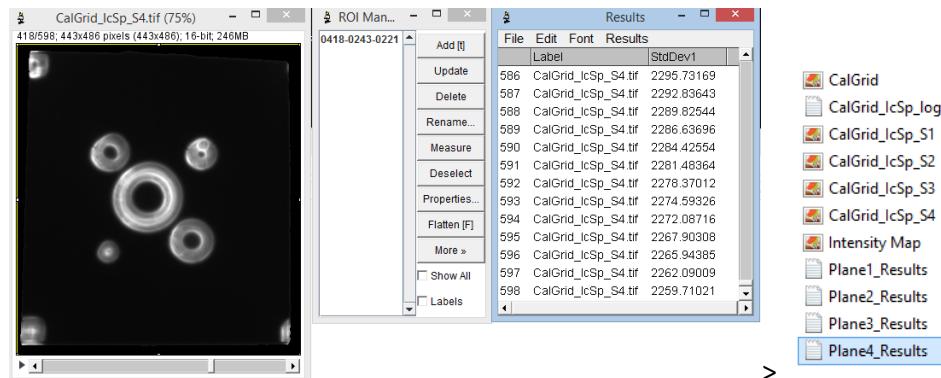


- Click on the image of plane 3, click on the selection in the ROI Manager, launch in the ROI Manager More >> Multi Measure..., select in the emerging dialog the options “Measure all ... slices” and “One row per slice”, and press OK, Save the emerging results window as a text file to the directory (e.g. under the name “Plane3_Results.txt”) viaFile > Save As...

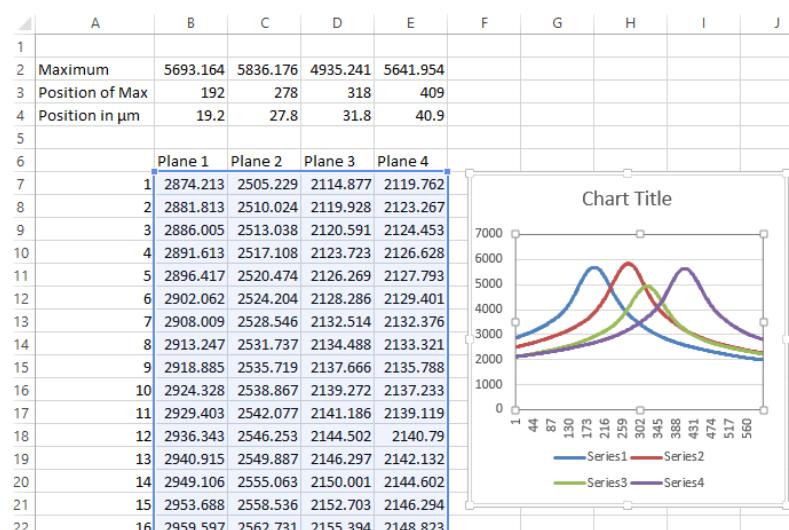
More information, license notes, credits: <https://github.com/hansenjn/MultifocalImaging-AnalysisToolbox>



- Click on the image of plane 4, click on the selection in the ROI Manager, launch in the ROI Manager More >> Multi Measure..., select in the emerging dialog the options “Measure all ... slices” and “One row per slice”, and press OK, Save the emerging results window as a text file to the directory (e.g. under the name “Plane4_Results.txt”) via File > Save As...

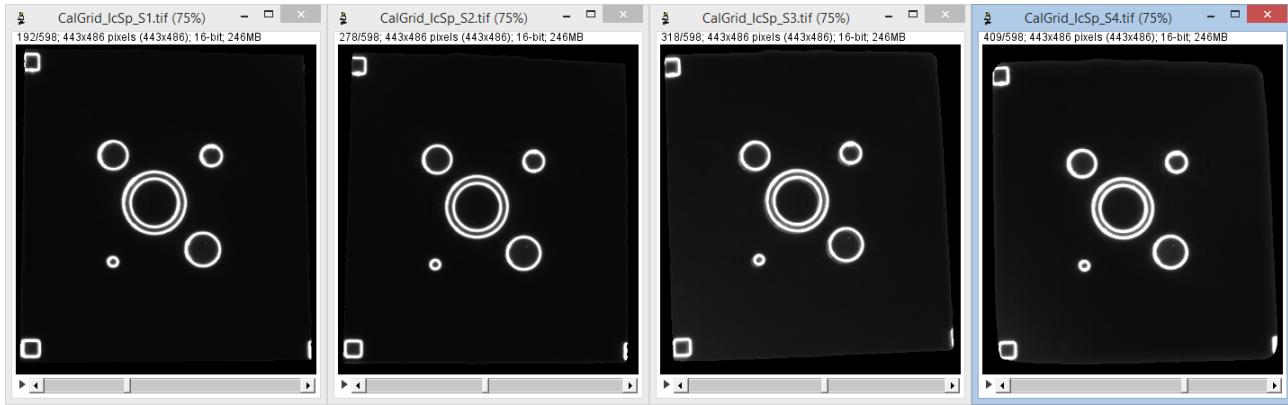


The text files are tab-delimited. Open the files and form a table showing the z step in the first column, standard deviation for plane 1 in the second column, standard deviation for plane 2 in the third column, standard deviation for plane 3 in the fourth column, standard deviation for plane 4 in the fifth column. For each plane, extract the z step positions where the standard deviation is maximal – this represents the focal position in each plane (the position where the focal plane maps the specimen most sharply). Note down these positions in micron (calculate them by multiplying the slice position with the step size of the piezo recording), they are required for calibration and analysis later in the workflow.

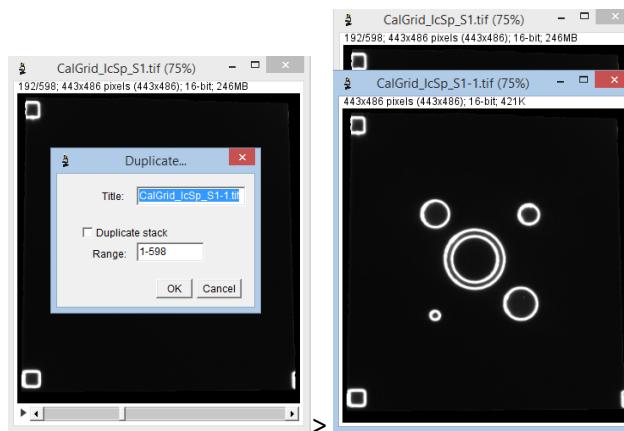


Extract the sharpest image from each plane and use it to generate an alignment file as follows:

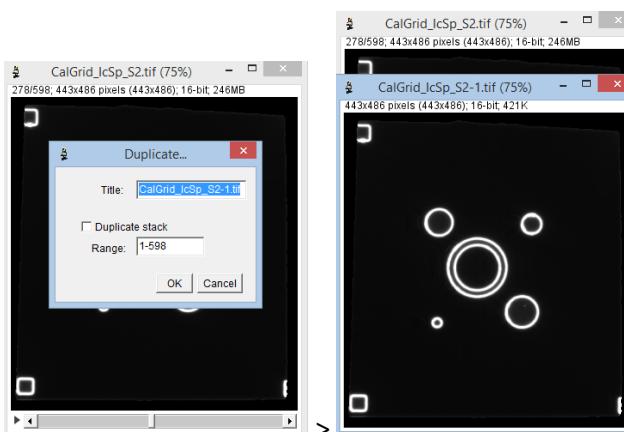
- Go to each plane image stack in ImageJ and move the slice bar to the position that maps the grid most sharply (this is the position of highest standard deviation which you determined above and noted down). For example, for plane 1 we determined above the position 192 as most sharp, thus here we moved to position 192 (see number written above the image on the left).



- Duplicate the most sharpest image for each time point:
 - Click on window showing plane 1, Image > Duplicate, make sure the “Duplicate stack” option is not checked, press OK.

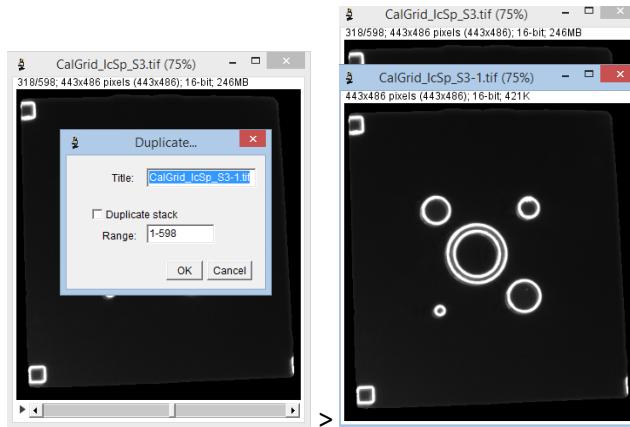


- Click on window showing plane 2, Image > Duplicate, make sure the “Duplicate stack” option is not checked, press OK.

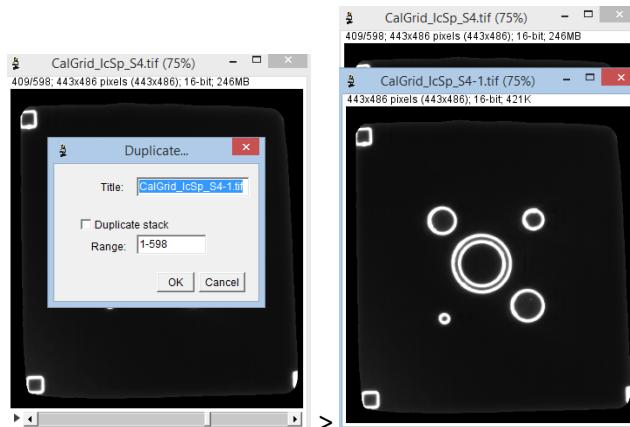


More information, license notes, credits: <https://github.com/hansenjn/MultifocalImaging-AnalysisToolbox>

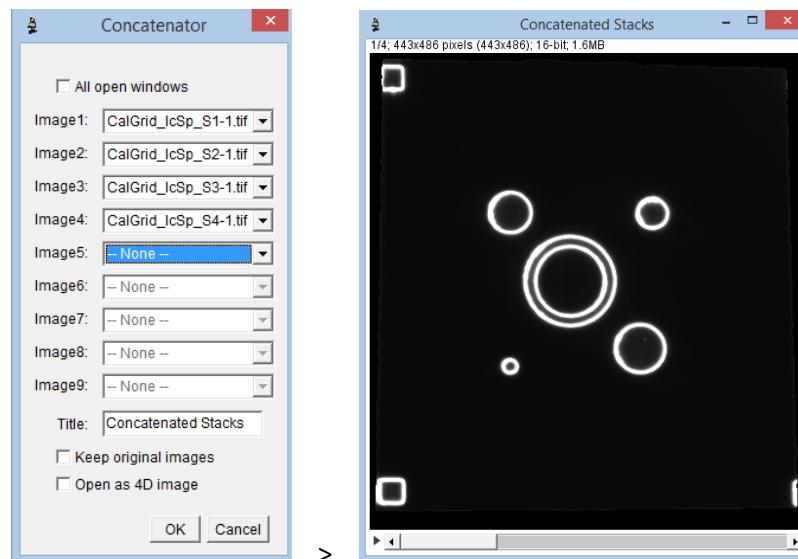
- Click on window showing plane 3, Image > Duplicate, make sure the “Duplicate stack” option is not checked, press OK.



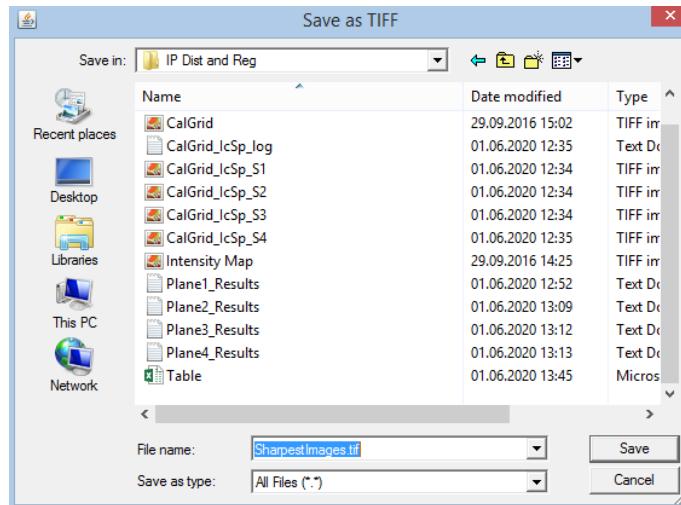
- Click on window showing plane 4, Image > Duplicate, make sure the “Duplicate stack” option is not checked, press OK.



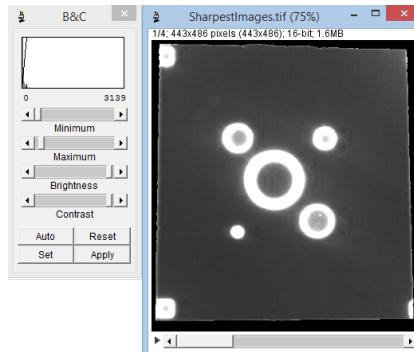
- Concatenate the duplicates to a stack: Image > Stack > Tools > Concatenate..., select the duplicates for planes 1 to 4 as Image1 to Image4, respectively, click OK.



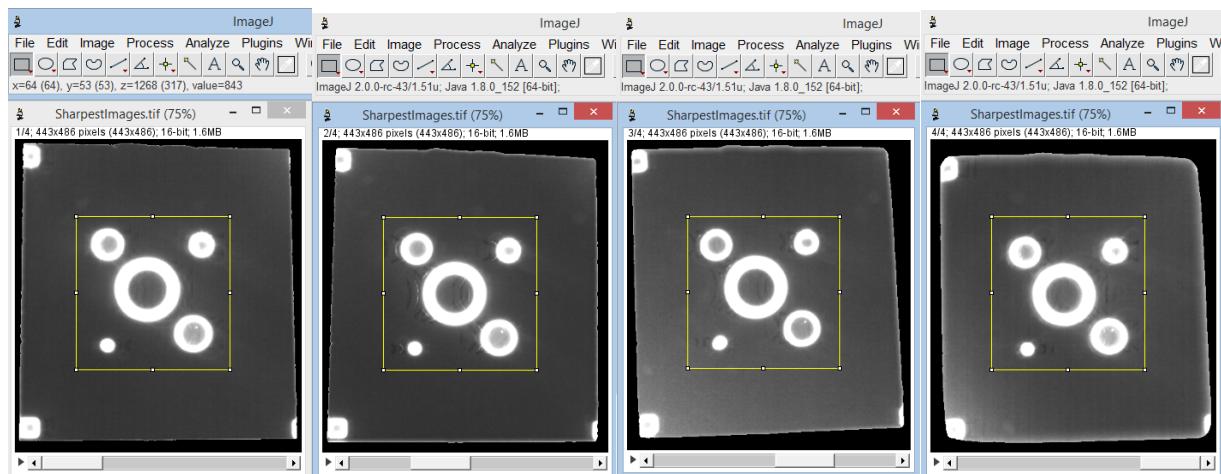
- Save the image for documentation: File > Save As > Tiff...



- 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

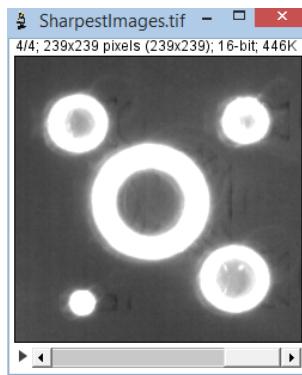


- Select the Rectangle tool in the ImageJ bar and draw a selection that is inside all planes and does not contain any black areas. Check the different planes by scrolling with the mouse wheel and adapting the selection.

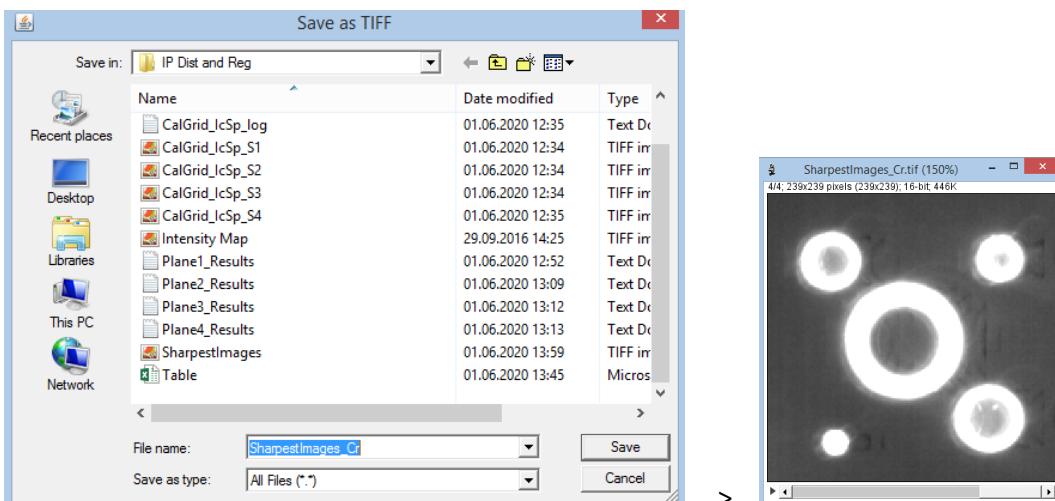


- Crop the stack to the selection: Image > Crop

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

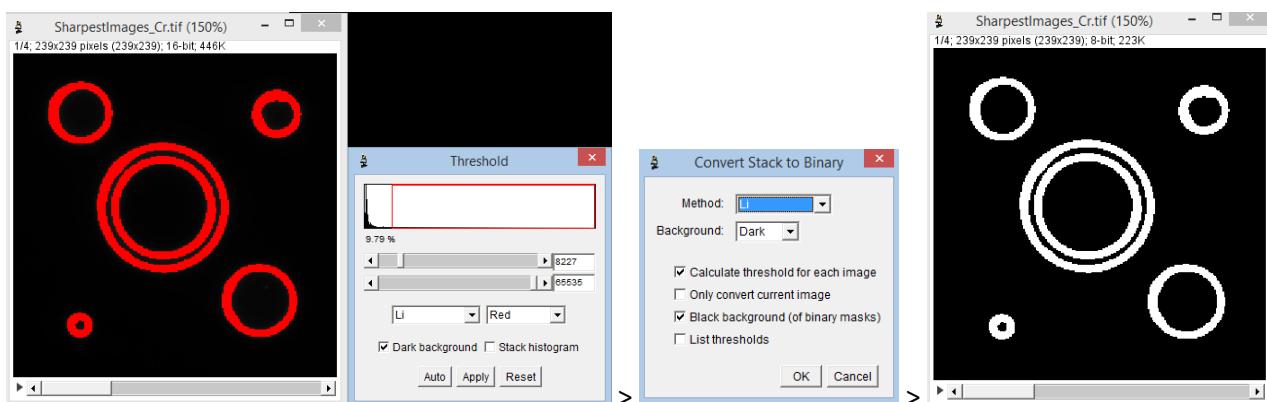


- Save the stack as RegistrationTemplate_IcSp_Cr: File > Save As ... > Tiff



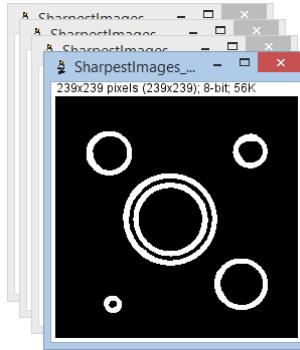
If you do not use a FIJI distribution of ImageJ, you need to install <https://imagej.net/Skeletonize3D> to your ImageJ to perform the next steps. In the FIJI distribution of ImageJ you can perform the next steps as described without any additional installation:

- Launch FIJI and open the image you just saved (called SharpestImages_Cr above).
- Binarize the image: Image > Adjust > Threshold, select settings shown below, click Apply, select settings shown below in the emerging dialog and click OK.



- Split the Stack into Images: Images > Stacks > Stack to Images

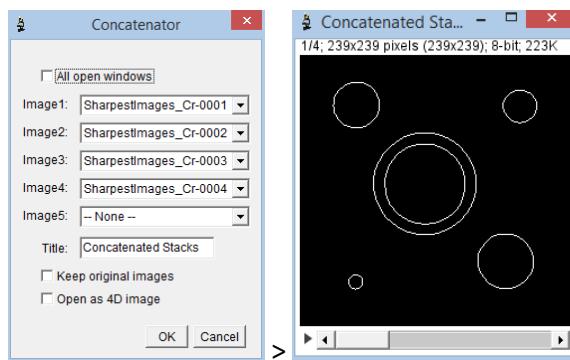
More information, license notes, credits: <https://github.com/hansenjn/MultifocalImaging-AnalysisToolbox>



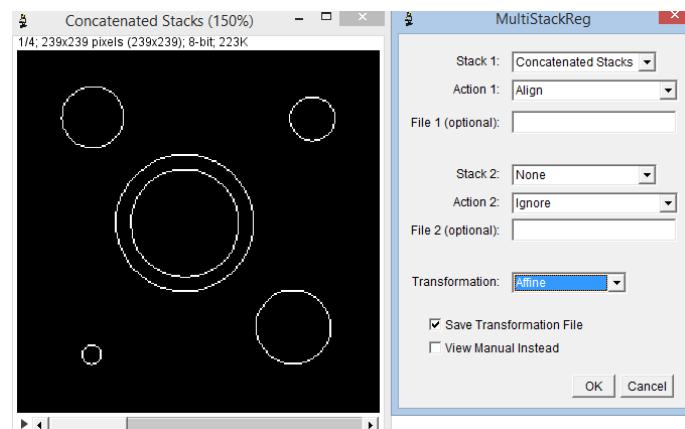
- Convert each of the Image to a Skeleton: for each image, click on the image window and go to Plugins > Skeleton > Skeletonize (2D/3D)



- Pack the individual images into a stack again: Images > Stacks > Tools > Concatenate..., set the Images with endings 0001 to 0004 as Image1 to Image4, respectively.

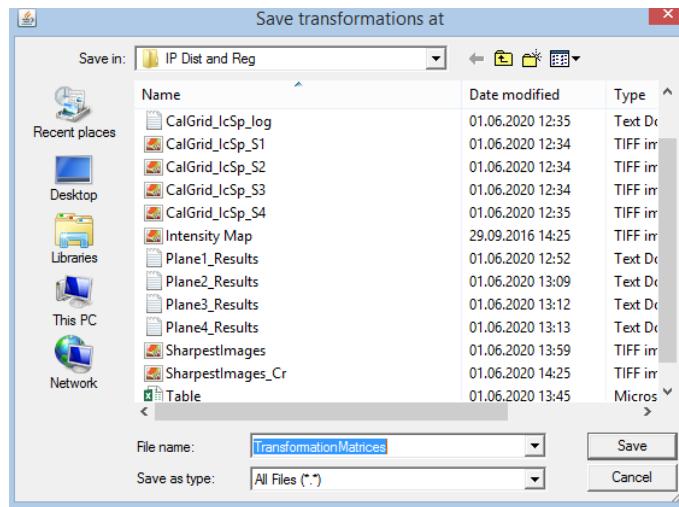


- Register the file: Plugins > Registration > MultiStackReg, select the following settings, press OK



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

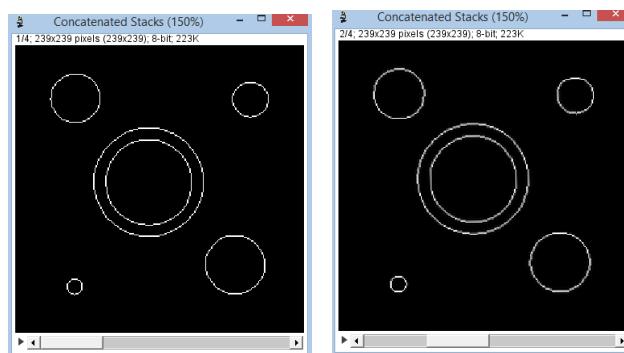
- A dialog pops up, save the transformation matrices to the folder where the image was located



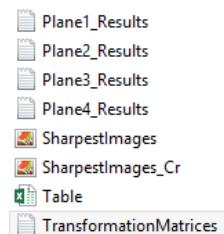
- Wait until the plugin is done and the status bar looks “resting”/normal again



- 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

Perform the same procedure as described in the section “Preparing the data for calibration and analysis” of the chapter “3D Bead tracking” (see above). Use the “TransformationMatrices” and the “Intensity Map.tif” specific to the flagellar recordings that were generated in the previous two chapters.

Determine a calibration Look-Up-Table (LUT)

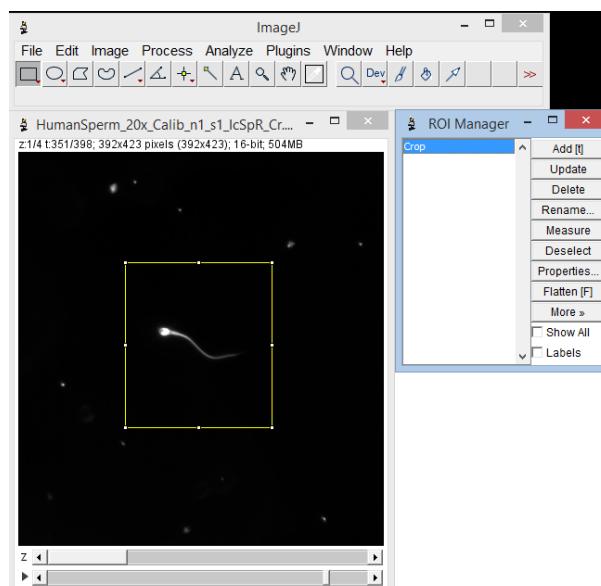
Prepare multifocal z-stacks through non-moving flagella with defined, constant z-step size and prepare the generated image stacks as described in the previous chapter. Next, proceed with the images to SpermQ-MF calibration analysis as follows.

For each sperm to be included in the calibration LUT, crop the image to the individual sperm cell as follows to generate images that contain only one sperm cell (like [SpermQ](#), SpermQ-MF can only process one sperm per image; in addition, cropping to a smaller region of interest removing blank and unnecessary image regions speeds up the SpermQ-MF processing):

- Open the Image in ImageJ and move to a piezo position where you can clearly see the flagellum.



- Draw a rectangular ROI around the sperm cell, leaving sufficient space for normal vector generation around the sperm cell (normal vector generation is described in the [publication of SpermQ](#), normal vectors are depicted in Fig. 2C). For documentation, you may transfer the ROI to the ROI Manager by pressing **t** on the keyboard, and save the ROI in the ROI Manager to your file directory via More >> Save... (e.g. under the name “Crop”).

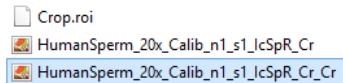


- Crop the Image to the ROI's size: Image > Crop

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



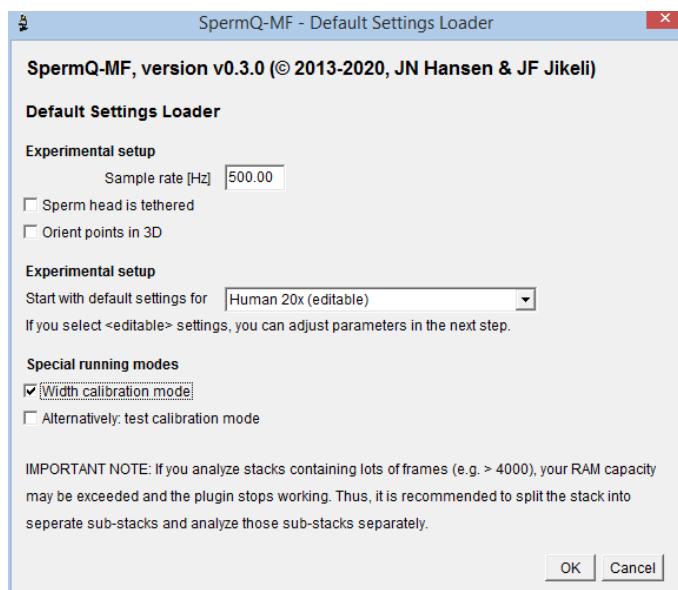
- Save the image under the image's name + ending “_Cr”: File > Save As > Tiff...



- Close the Image.
- **Note:** you may also crop multiple sperm from the same recording – then, give the cropped images different name endings to not overwrite (e.g. “..._Cr_1.tif”, “..._Cr_2.tif”, etc.)

Once you have generated all individual sperm images that you want to include in the calibration LUT, Measure the width of the flagellum in all images using SpermQ-MF:

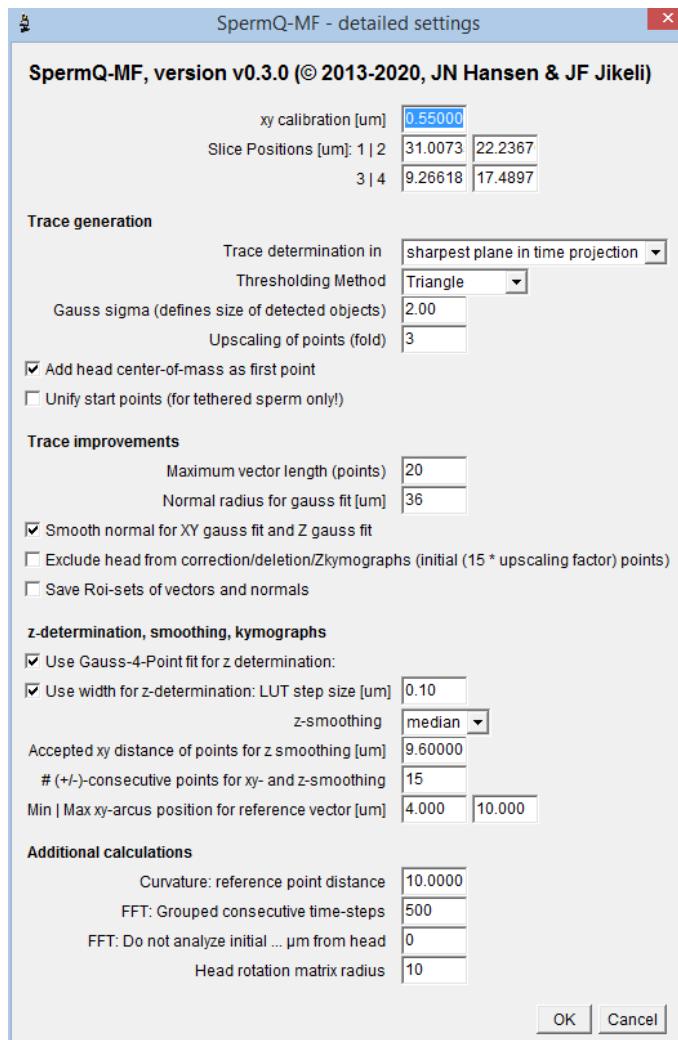
- Eventually, install SpermQ-MF (needs to be performed only once, before the first time use of the plugin in the ImageJ distribution):
 - Open ImageJ
 - Download SpermQ-MF (<https://github.com/hansenjn/SpermQ-MF/releases>)
 - Drag and drop the .jar file into your ImageJ window for installation and press “Save” in the upcoming dialog
 - Restart ImageJ
- Launch SpermQ-MF: Plugins > JNH > Multi Focal > SpermQ-MF Analysis, set the following settings



- Notes on the settings:
 - When the special running mode “Width calibration mode” is selected, the settings “Sample rate”, “Sperm head is tethered”, and “Orient points in 3D” are obsolete and do not need to be adapted.

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

- Select “default settings” that fit best to the experimental paradigm that you apply and select editable to be able to adapt them to your paradigm.
- A dialog pops up that allows to edit the analysis settings, select the settings as follows, while adapting few settings according to your setup as described below, then click OK.



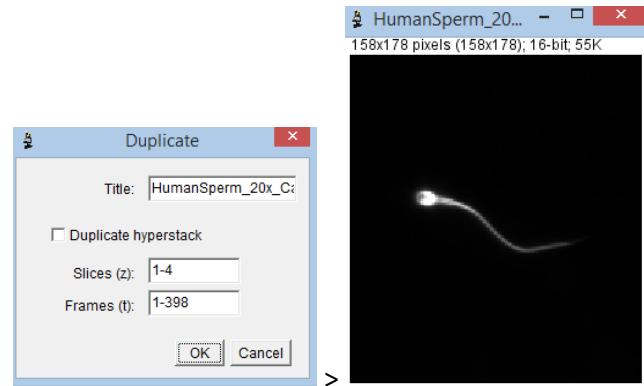
- Notes on the settings:

- **xy calibration:** enter here the calibration of your image
- **Slice Positions:** obsolete in width calibration mode
- **Thresholding Method** and **Gauss sigma:** These parameters determine how the trace is detected. They may need optimization according to your images. Briefly, SpermQ-MF will extract the sharpest image in your stack to retrieve flagellar coordinates by blurring the image with a Gaussian blur (you can adapt this process by adapting the “Gauss sigma”), binarize the image (you can adapt the binarization threshold by adapting the “Thresholding method”), blur the binarized image again with the same Gauss filter as before binarization, skeletonize the image, and retrieve the flagellar points from the skeleton as described in detail in the [SpermQ publication](#). You may manually check which algorithm works best as follows:
 - Open the image
 - Move to a position in the stack where the image sharply maps the sperm cell

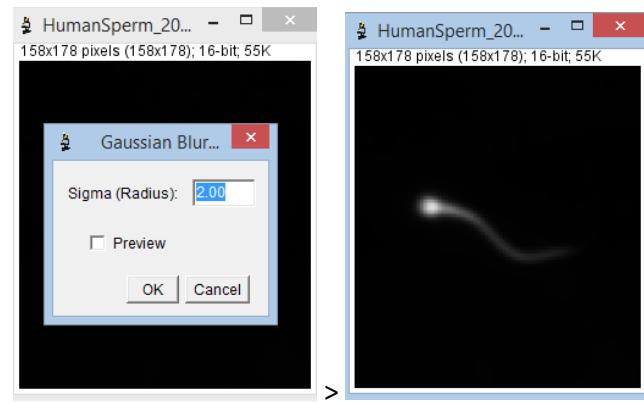
More information, license notes, credits: <https://github.com/hansenjn/MultifocalImaging-AnalysisToolbox>



- Duplicate the image: Image > Duplicate, unselect “Duplicate Hyperstack” option, click OK

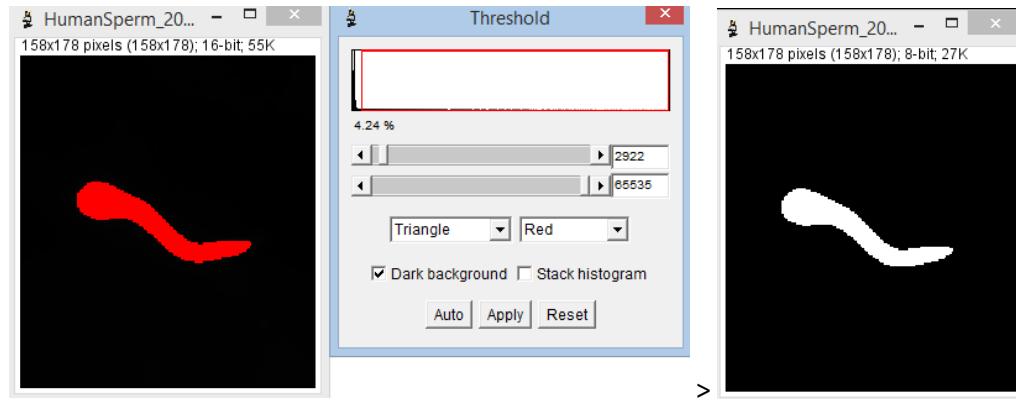


- Blur the image as you would set in SpermQ-MF settings: Process > Filters > Gaussian Blur; in the emerging dialog, select the Sigma that you aim to use in SpermQ-MF settings, click OK



- Open the Thresholding window: Image > Adjust > Threshold..., select the threshold that you wish to select in SpermQ-MF, Click Apply,

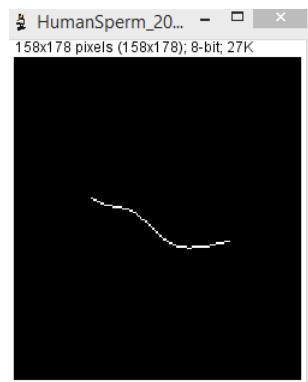
More information, license notes, credits: <https://github.com/hansenjn/MultifocalImaging-AnalysisToolbox>



- Blur the image again as you would set in SpermQ-MF settings: Process > Filters > Gaussian Blur; in the emerging dialog, select the Sigma that you aim to use in SpermQ-MF settings, click OK

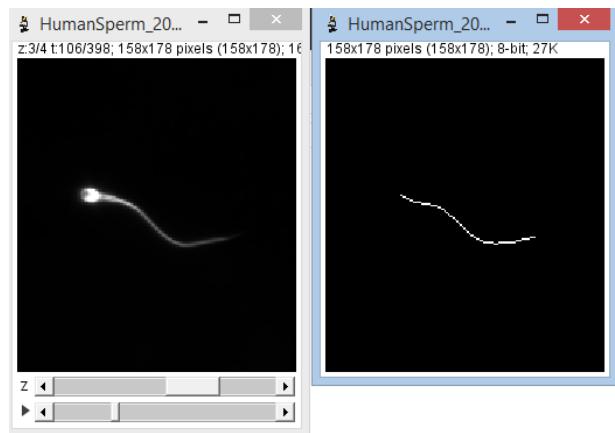


- Skeletonize the image: Plugins > Skeleton > Skeletonize (2D/3D) (if this option does not exist in your ImageJ menu, install the ImageJ plugin <https://imagej.net/Skeletonize3D> or use a <https://imagej.net/Fiji/Downloads> distribution, where the plugin is already preinstalled)

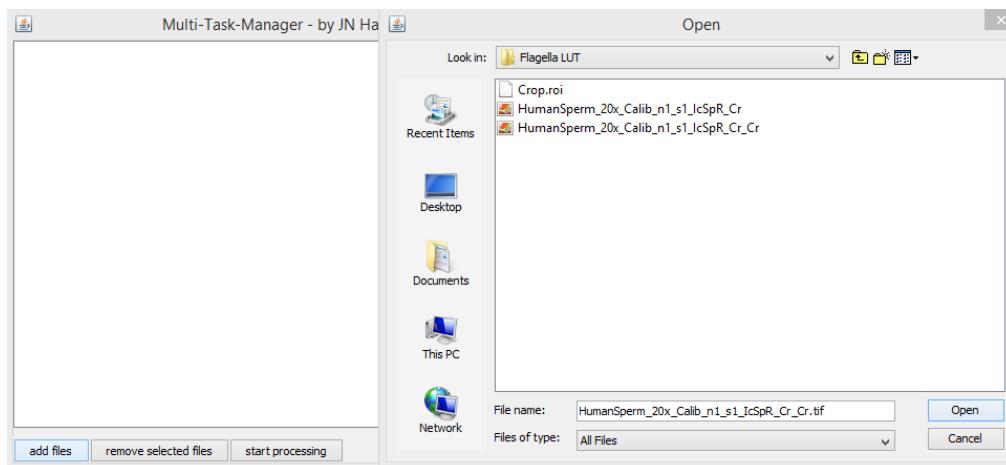


- Compare the image to the unprocessed image to see whether tracking is ok (the skeleton is in the center of the flagellum) or not (no skeleton produced, skeleton not tracking the whole flagellum, side branches, etc.).

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- If the tracking is not “ok”, you may alter the Gaussian Blur and Thresholding Settings in these manual steps until you achieve a good tracking. Note down the settings that give the best tracking and use them in SpermQ-MF.
- Settings for **Trace improvements** are precisely described and explained in the [SpermQ publication](#)
- All other settings are obsolete in the **width calibration mode**
- A dialog emerges asking you to load files that you want to process. Add all sperm images that you want to use to generate the LUT in to the files list and press “start processing”.



- For every image you listed for analysis, now a maximum projection pops up and a dialog asking you to set a ROI. This ROI helps SpermQ-MF to find the sperm cell in the image, set a ROI around at least the head or around the whole sperm cell (should any additional particles are shown by the image, make sure they are not included in your ROI) and press OK.

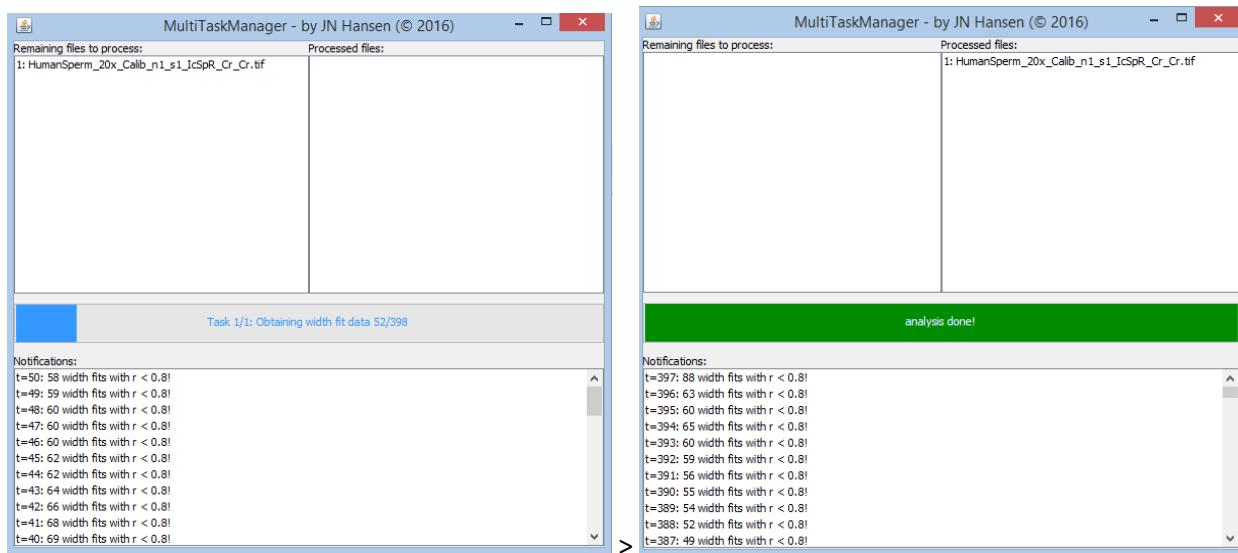


- Next, for every image you listed for analysis, now a maximum projection pops up and a dialog asking you to set another ROI. Draw a ROI where the whole cell is contained but not any other particle that might be in the image than the sperm cell. This ROI will determine the region in which SpermQ-MF determine the standard deviation (SD) across the stack to find the most sharpest (highest SD) image that will in turn be used to retrieve flagellar points. After drawing a ROI, click OK.

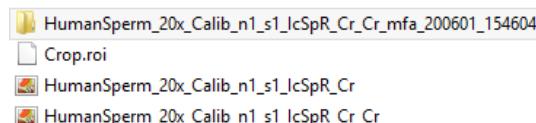


- Wait until the MultiTaskManager states “analysis done”. Commonly many log entries emerge during processing, these are just for debugging the software and can be ignored. The analysis can take a couple of minutes per file. If you have listed many files this can take quite a while. However, during analysis (after you have set all ROIs in the beginning) no user interaction is required, so you can also start the analysis in the evening and let it run overnight.

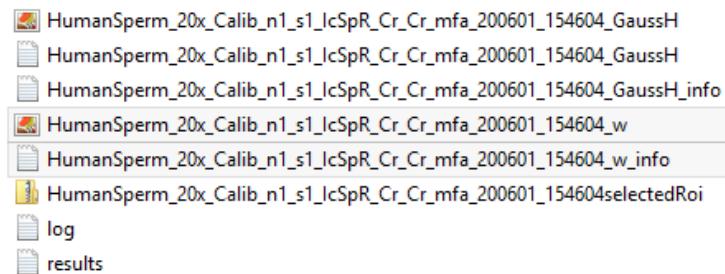
More information, license notes, credits: <https://github.com/hansenjn/MultifocalImaging-AnalysisToolbox>



- For each analyzed image, SpermQ-MF creates a directory in the directory where the analyzed image was placed. This directory contains the analyzed image's name and the ending “_mfa_<analysis date in form yyyyymmdd_hhmmss>”.

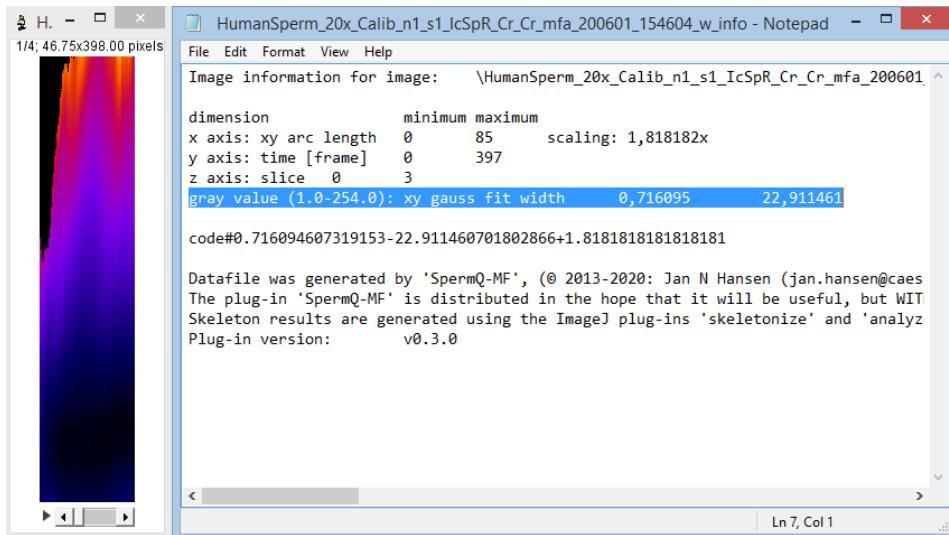


- Open the directory.



- The files with ending “_w.tif” and “_w_info.txt” need to be extracted for LUT generation. The tif file describes the flagellar width, encoded as an intensity value (x coordinate: arc length on the flagellum, y coordinate: piezo position). The intensity value can be decoded to a flagellar width value based on the description in the _w_info.txt file.

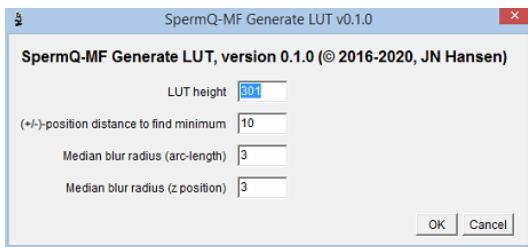
More information, license notes, credits: <https://github.com/hansenjn/MultifocalImaging-AnalysisToolbox>



- **DO NOT** modify these files because they need to be used in LUT generation as output by SpermQ-MF!

When you have obtained the width profiles from all flagella you want to use for generating a look-up-table (LUT) that allows to infer the z-position from the flagellar width, use SpermQ-MF GenerateLUT to generate the LUT:

- Launch ImageJ
- Eventually, install SpermQ-MF_GenerateLUT (needs to be performed only once, before the first time use of the plugin in the ImageJ distribution):
 - Open ImageJ
 - Download SpermQ-MF (https://github.com/hansenjn/SpermQ-MF_GenerateLUT/releases)
 - Drag and drop the .jar file into your ImageJ window for installation and press “Save” in the upcoming dialog
 - Restart ImageJ
- Launch SpermQ-MF GenerateLUT: Plugins > JNH > Multi Focal > SpermQ-MF Generate LUT, select the following settings (explained below), press OK.

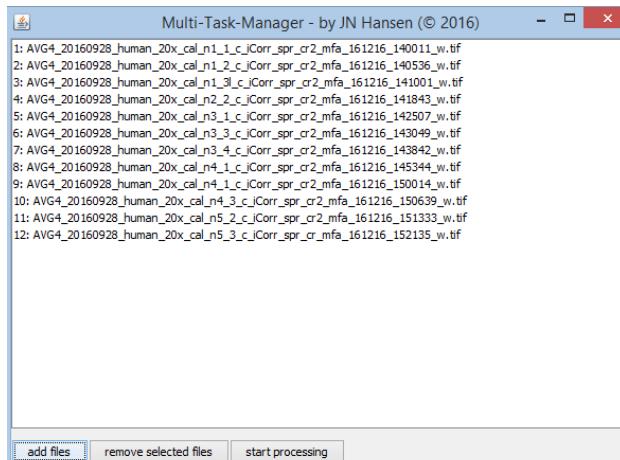


- **LUT height:** defines the height of the LUT that you want to generate. E.g. for a value of 301, you will create the LUT for 150 piezo steps above and 150 piezo steps below the actual z-position of the flagellum.
- **(+/-)-position distance to find minimum:** determines how the minimum width, i.e. the actual z-position of the flagellum is found. This value indicates how many consecutive piezo steps are included into a sliding window that is shifted at each arc length position along the piezo positions to find the region with minimal width value, i.e. the actual z-position of the sperm cell.
- **Median blur radius:** When for each input width file and at each arc length position, a minimum is determined, all input width files are overlaid and aligned so that minimum

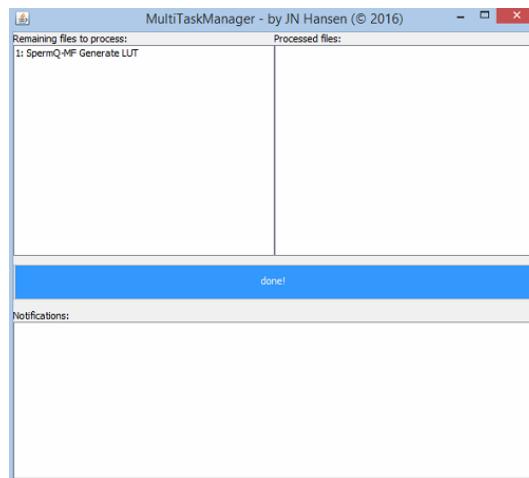
More information, license notes, credits: <https://github.com/hansenjn/MultifocalImaging-AnalysisToolbox>

positions overlap. Next, all width files are averaged and the resulting LUT is smoothed with a Median filter. The kernel size used for the Median Filter is controlled by changing the **Median blur radius** parameters.

- A dialog emerges asking to load files. Add all width files that you generated with SpermQ-MF and press start processing.



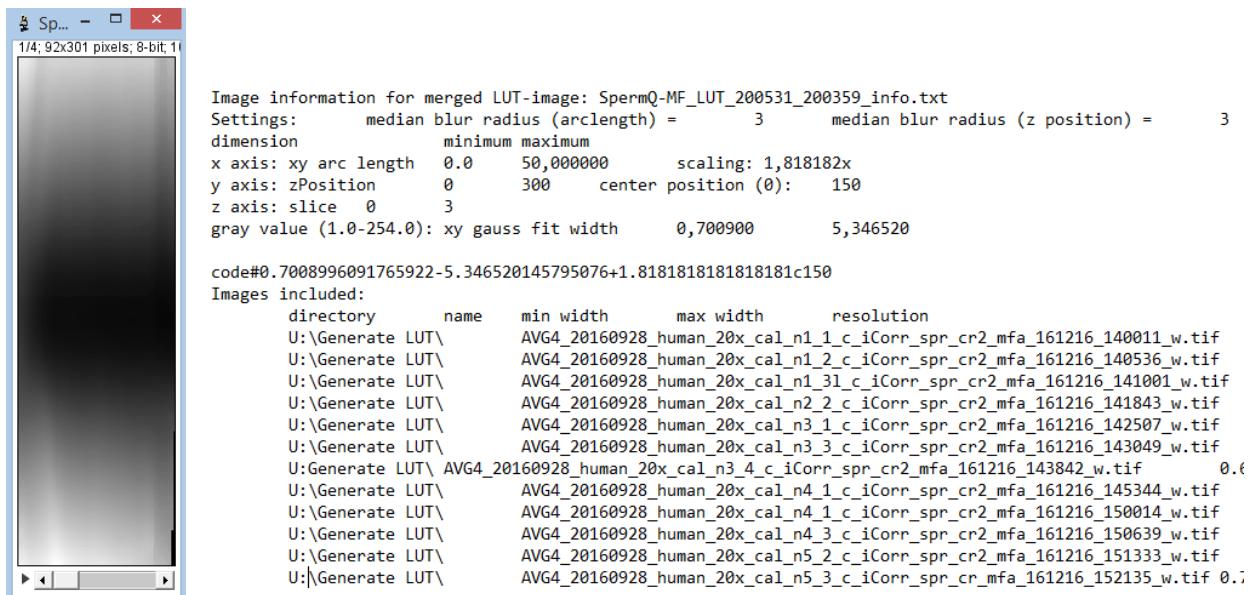
- Wait until the MultiTaskManager dialog states “done”



- Two files have been generated automatically in the directory of the first width file you added to the list. The tif file contains the LUT, the info file documents the settings and which width files were used in the LUT.



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



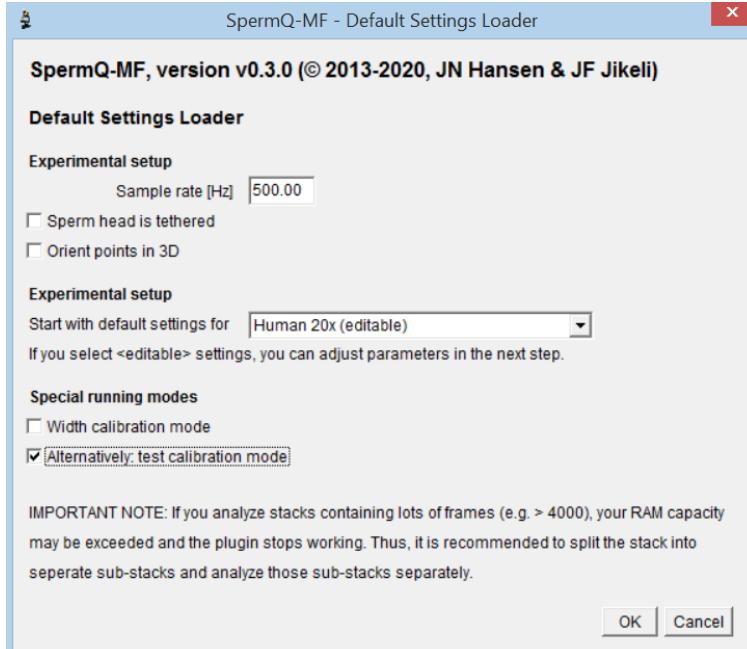
Verify the calibration Look-Up-Table (LUT)

Prepare multifocal z-stacks through non-moving flagella with defined, constant z-step size and prepare the generated image stacks as described in the previous chapter. Next, proceed with the images to SpermQ-MF calibration analysis as follows.

For each sperm to be included in the calibration LUT, crop the image to the individual sperm cell as described in the previous section.

Proceed to analysis of the files:

- Launch SpermQ-MF: Plugins > JNH > Multi Focal > SpermQ-MF Analysis, set the following settings

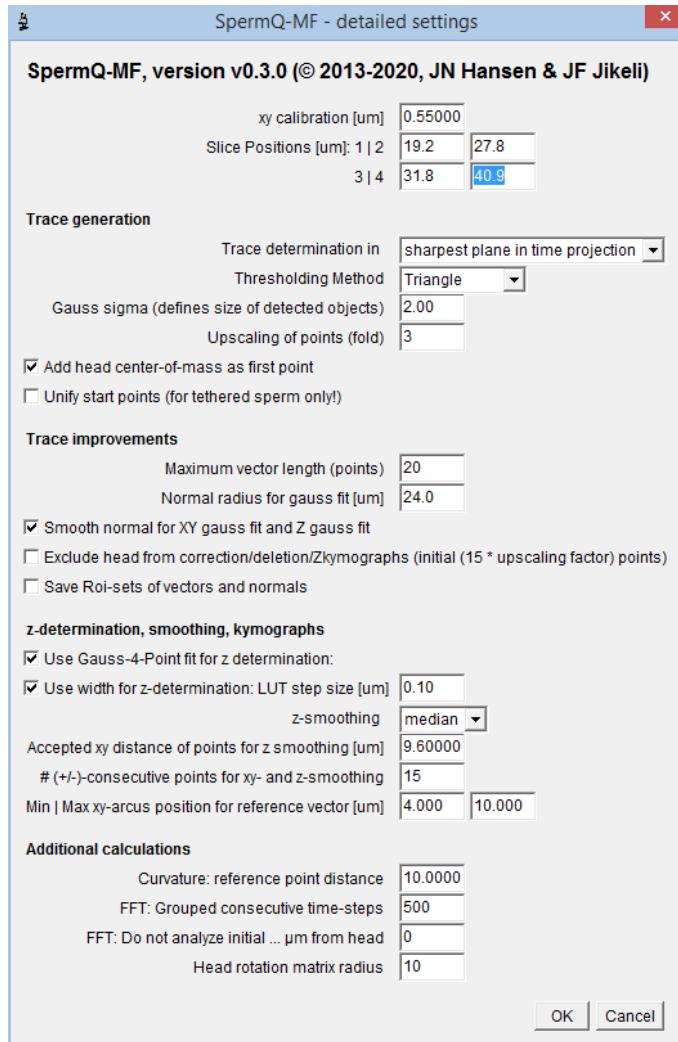


- Notes on the settings:

- When the special running mode “test calibration mode” is selected, the settings “Sample rate”, “Sperm head is tethered”, and “Orient points in 3D” are obsolete and do not need to be adapted.

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

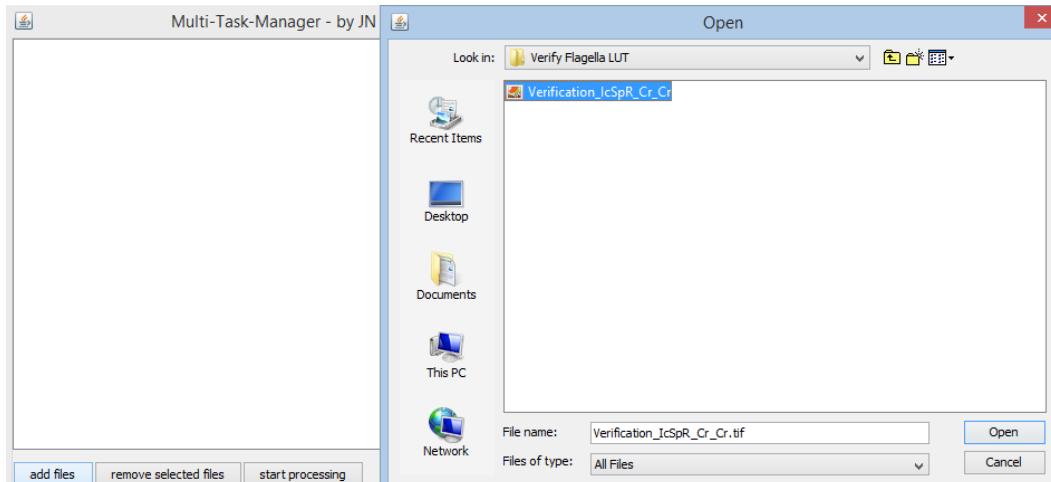
- Select “default settings” that fit best to the experimental paradigm that you apply and select editable to be able to adapt them to your paradigm.
- A dialog pops up that allows to edit the analysis settings, select the settings as follows, while adapting few settings according to your setup as described below, then click OK.



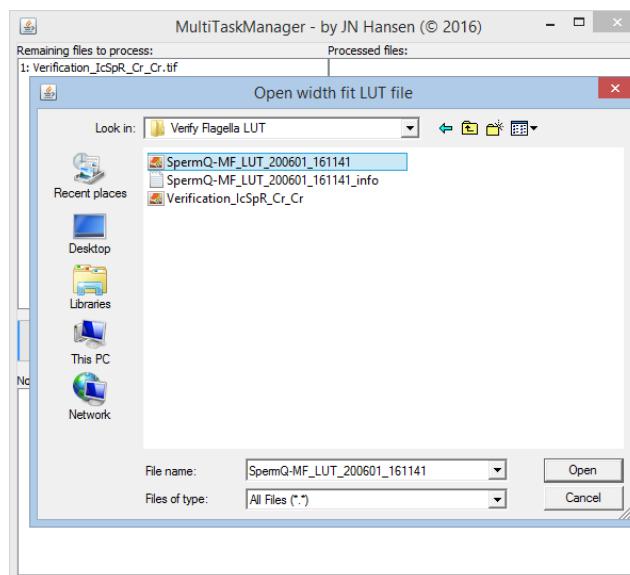
- **Slice positions:** enter here the positions (for planes 1,2,3,4) that you determined according to section “Creating a registration file & determining focal positions” (see above).
- **xy calibration, trace generation, trace improvements:** enter the settings identical to what you set in the previous section for generating width files used for the LUT.
- **Z-determination, interpolation and kymographs.**
 - Keep both first checkboxes checked
 - Indicate the LUT step size = the y-length of one pixel in the LUT: this corresponds to the step size of the piezo during recording of the calibration images.
 - “Min | Max xy-arcus position...”, these two parameters define the range of arc-length positions on the flagellum that are used to determine the orientation vector of the cell in space, and thereby, the angle theta. See also the [SpermQ publication](#) for more details on the orientation vector and angle theta.
 - The other settings are analogous to SpermQ settings – they are precisely described and explained in the [SpermQ publication](#)

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

- **Additional calculations:** the settings are precisely described and explained in the [SpermQ publication](#)
- A dialog emerges: add the prepared files you aim to use for verification and press “start processing”:

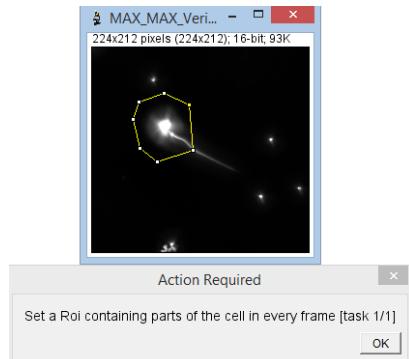


- A dialog emerges asking you to open the LUT file. Select the LUT file according to the previous section and click “Open”.

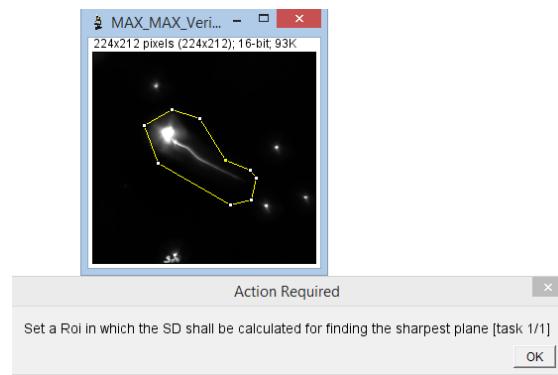


- For every image you listed for analysis, now a maximum projection pops up and a dialog asking you to set a ROI. This ROI helps SpermQ-MF to find the sperm cell in the image, set a ROI around at least the head or around the whole sperm cell (should any additional particles are shown by the image, make sure they are not included in your ROI) and press OK.

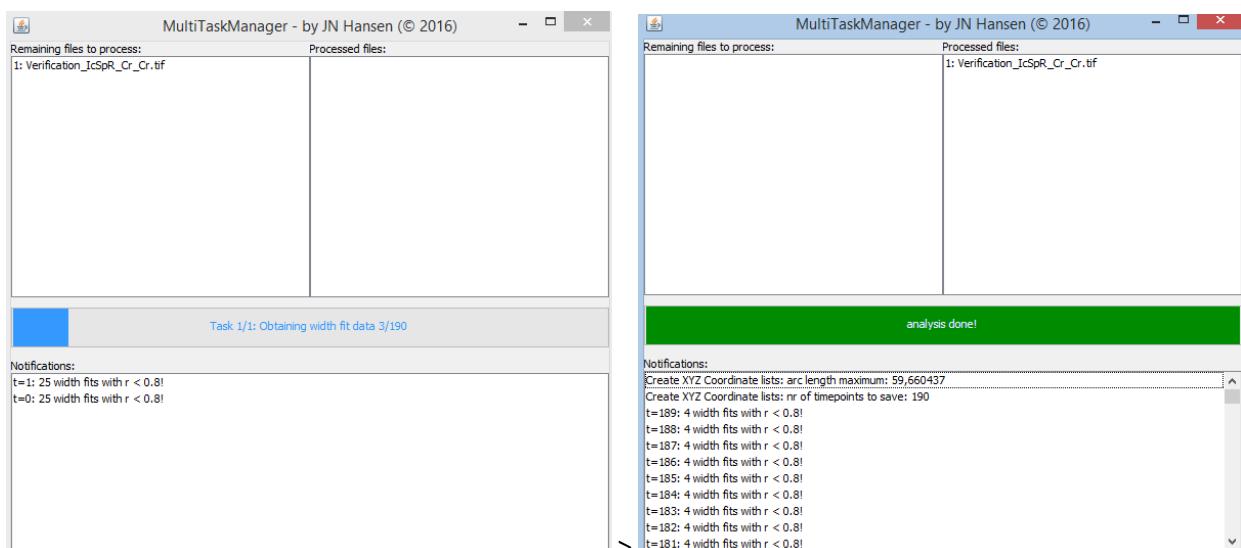
More information, license notes, credits: <https://github.com/hansenjn/MultifocalImaging-AnalysisToolbox>



- Next, for every image you listed for analysis, now a maximum projection pops up and a dialog asking you to set another ROI. Draw a ROI where the whole cell is contained but not any other particle that might be in the image than the sperm cell. This ROI will determine the region in which SpermQ-MF determine the standard deviation (SD) across the stack to find the most sharpest (highest SD) image that will in turn be used to retrieve flagellar points. After drawing a ROI, click OK.

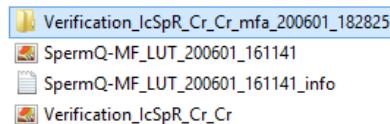


- Wait until the MultiTaskManager states “analysis done”. Commonly many log entries emerge during processing, these are just for debugging the software and can be ignored. The analysis can take a couple of minutes per file. If you have listed many files this can take quite a while. However, during analysis (after you have set all ROIs in the beginning) no user interaction is required, so you can also start the analysis in the evening and let it run overnight.

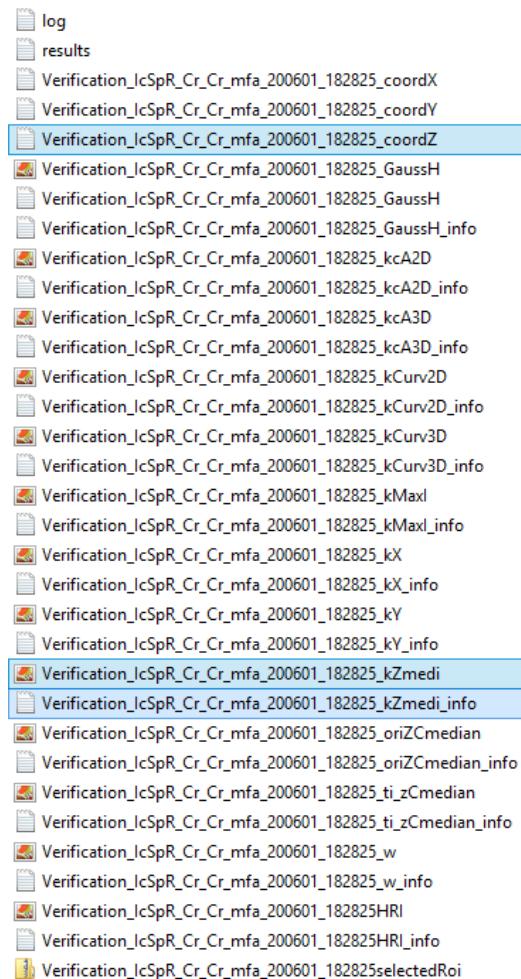


More information, license notes, credits: <https://github.com/hansenjn/MultifocalImaging-AnalysisToolbox>

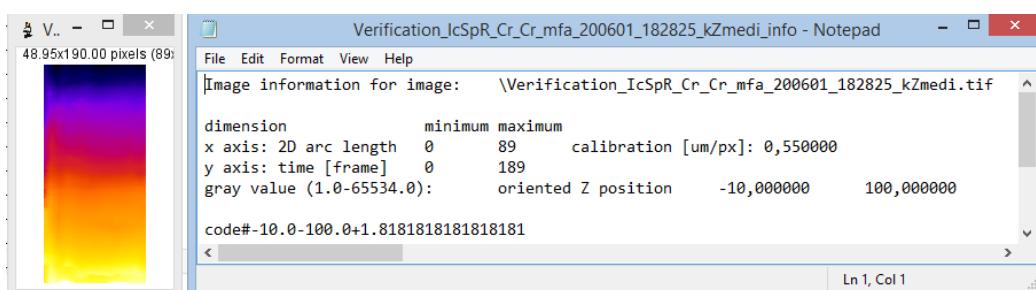
- For each analyzed image, SpermQ-MF creates a directory in the directory where the analyzed image was placed. This directory contains the analyzed image's name and the ending “_mfa_<analysis date in form yyyyymmdd_hhmmss>”.



- Open the directory.

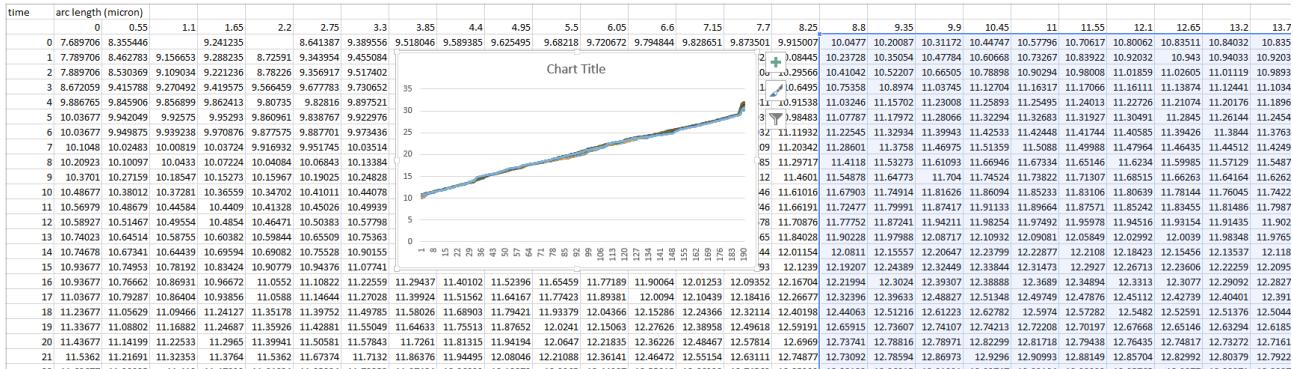


- The file with ending “KZmedi.tif” shows the z-positions (intensity-coded) determined along the arc length (x dimension) and along the piezo position (y dimension). The intensity-z-position-code can be retrieved from the file with ending “kZmedi_info.txt”.



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

- You may also retrieve the Z positions as a list from the file with ending coordZ.txt (it is tab-delimited).



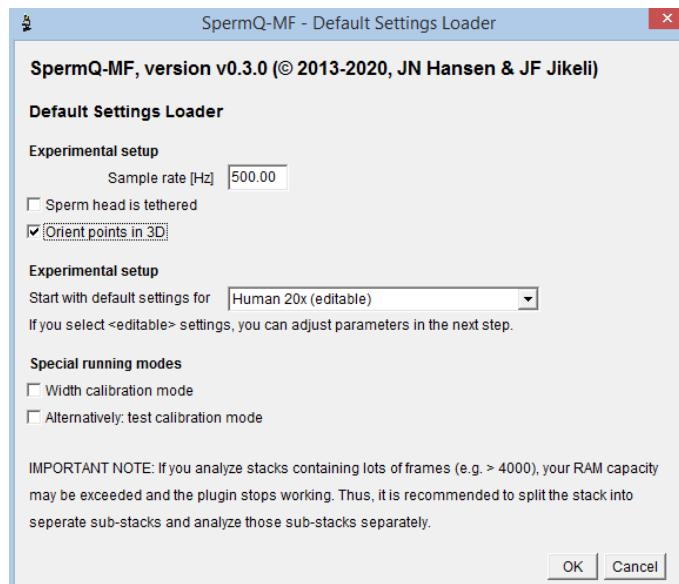
- The inferred z positions in relationship to the piezo position (indicated by the values in column "time" if multiplied by the step-size of the piezo during recording) should reveal a linear relationship with a slope of unity. The standard deviation of the residuals from a linear fit with slope unity 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.

3D reconstruction

When the setup has been calibrated by generating and verifying a LUT file (see previous two chapters), flagella can be analyzed from multifocal time-lapse recordings, acquired with exactly the same setup as the setup used to determine the LUT file. Prepare the image (as described in the chapter "Preparing the data for calibration and analysis"). Eventually, crop the files to smaller regions to reduce the amount of RAM used by the analysis (see beginning of section "Determine a calibration Look-Up-Table (LUT)").

Then proceed to SpermQ-MF analysis.

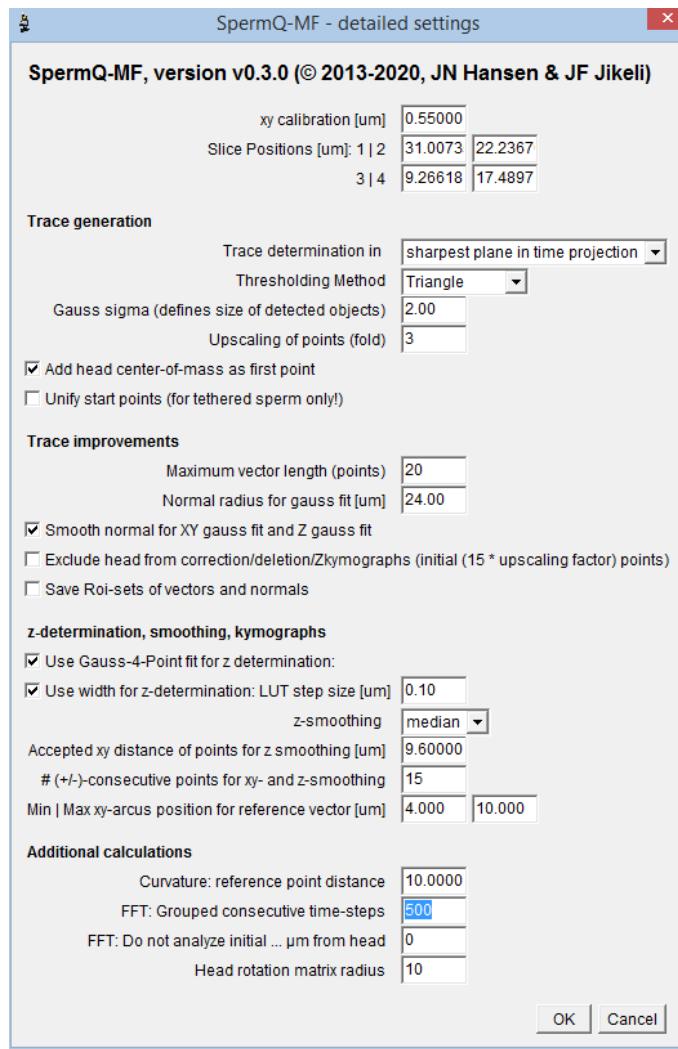
- Launch SpermQ-MF: Plugins > JNH > Multi Focal > SpermQ-MF Analysis, set the following settings



- Notes on the settings:

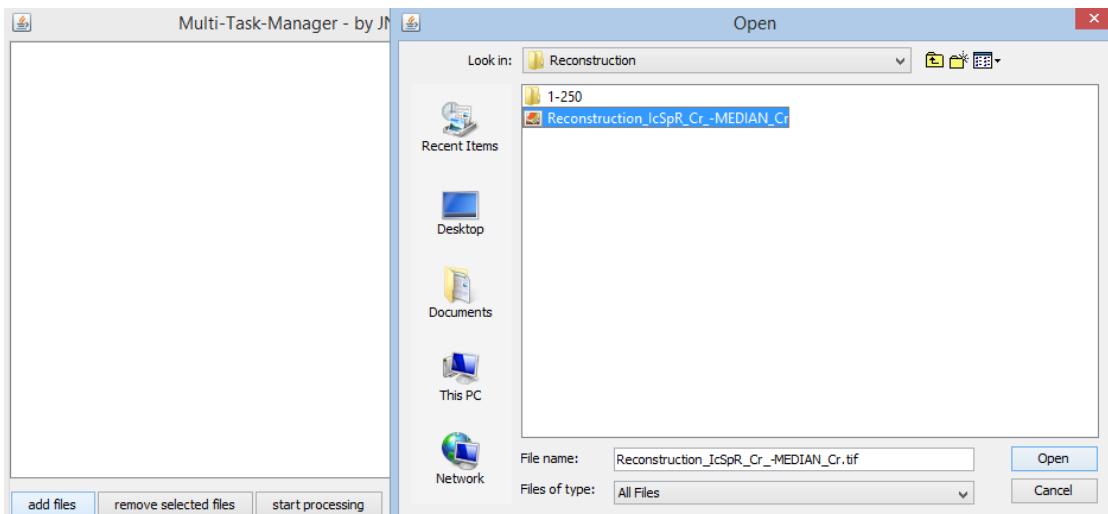
- Sample rate [Hz]:** enter the acquisition frequency of your recording, this information is required to calculate the flagellar beat frequency
- Sperm head is tethered:** check if you want to analyze head tethered sperm

- **Orient points in 3D:** If checked, a reference vector for determining the cell orientation based on the midpiece axis is calculated considering all three dimensions. See also the [SpermQ publication](#) for more details on the orientation vector and angle theta that is determined based on the orientation vector.
- Select **default settings** that fit best to the experimental paradigm that you apply and select editable to be able to adapt them to your paradigm.
- A dialog pops up that allows to edit the analysis settings, select the settings as follows, while adapting few settings according to your setup as described below, then click OK.

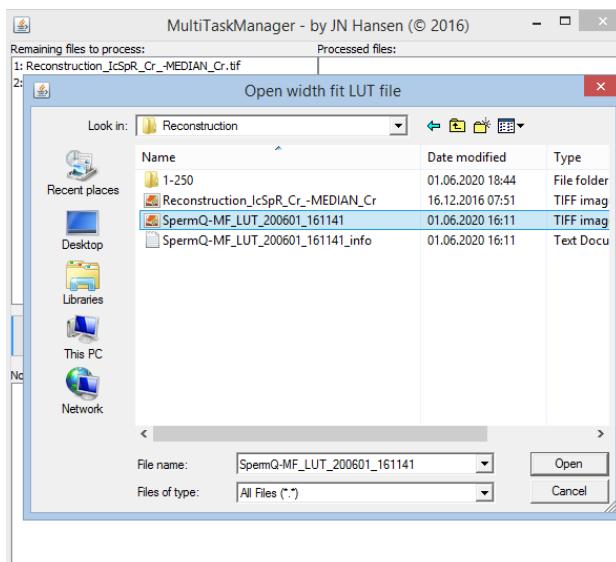


- **Settings:** Enter the same settings as used for determining and verifying the LUT (see two previous sections for more details on the settings).
- A dialog emerges: add the prepared files you aim to use for verification and press “start processing”:

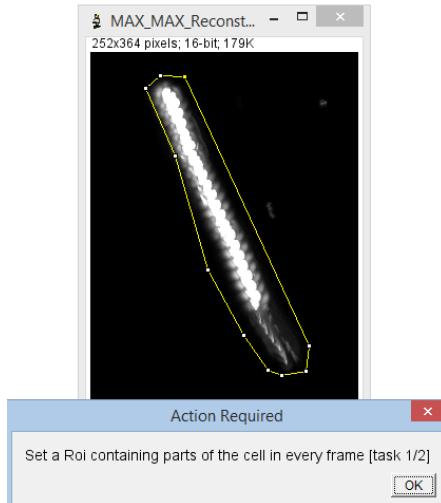
More information, license notes, credits: <https://github.com/hansenjn/MultifocalImaging-AnalysisToolbox>



- A dialog emerges asking you to open the LUT file. Select the LUT file according to the previous section and click “Open”.



- For every image you listed for analysis, now a maximum projection pops up and a dialog asking you to set a ROI. This ROI helps SpermQ-MF to find the sperm cell in the image, set a ROI around at least the head or around the whole sperm cell (should any additional particles are shown by the image, make sure they are not included in your ROI) and press OK.

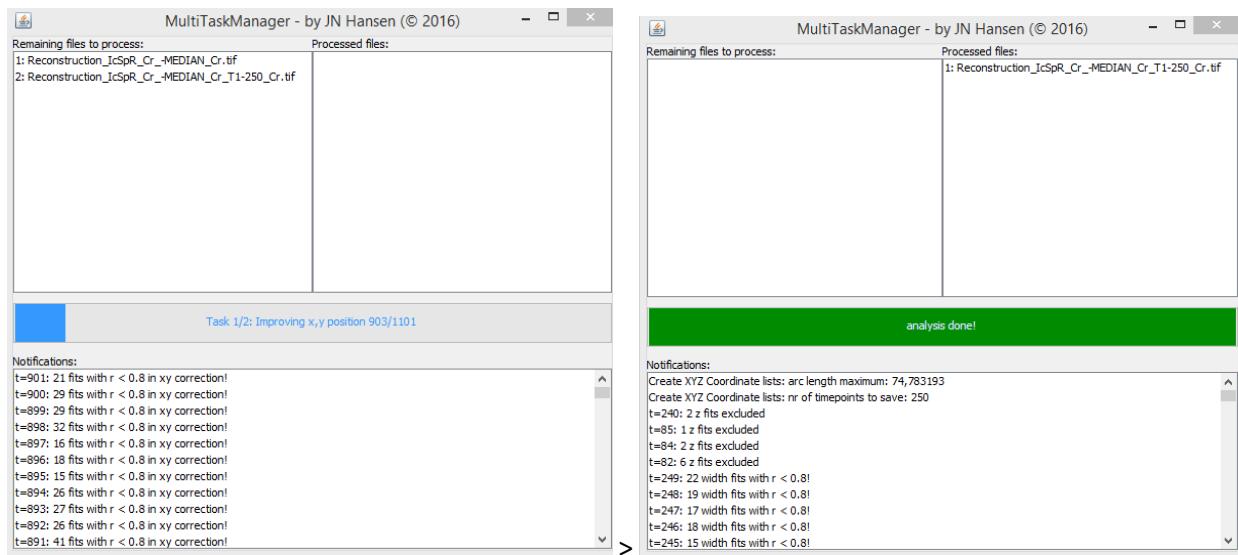


- Next, for every image you listed for analysis, now a maximum projection pops up and a dialog asking you to set another ROI. Draw a ROI where the whole cell is contained but not any other particle that might be in the image than the sperm cell. This ROI will determine the region in which SpermQ-MF determine the standard deviation (SD) across the stack to find the most sharpest (highest SD) image that will in turn be used to retrieve flagellar points. After drawing a ROI, click OK.

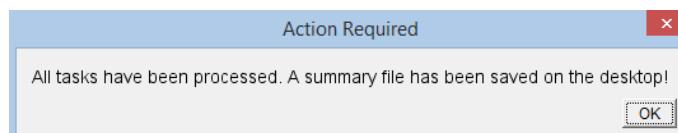


- Wait until the MultiTaskManager states “analysis done”. Commonly many log entries emerge during processing, these are just for debugging the software and can be ignored. The analysis can take a couple of minutes per file. If you have listed many files this can take quite a while. However, during analysis (after you have set all ROIs in the beginning) no user interaction is required, so you can also start the analysis in the evening and let it run overnight.

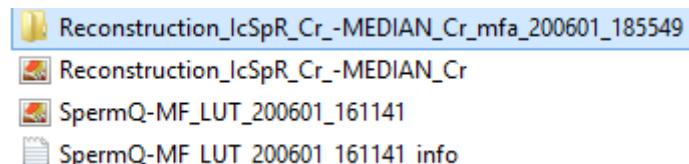
More information, license notes, credits: <https://github.com/hansenjn/MultifocalImaging-AnalysisToolbox>



- The plugin ends with throwing a dialog telling you that a summary file has been created on the desktop.

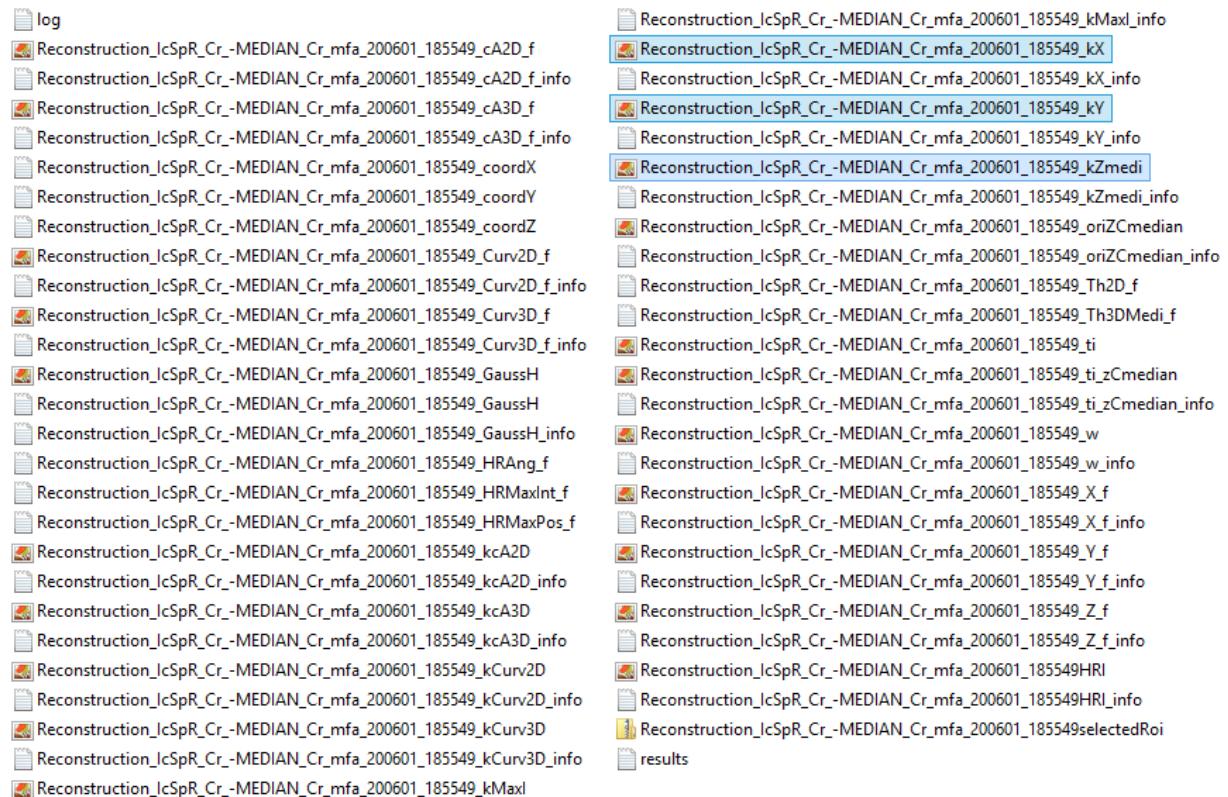


- The summary file is explained at the end of the section.
- For each analyzed image, SpermQ-MF creates a directory in the directory where the analyzed image was placed. This directory contains the analyzed image's name and the ending “_mfa_<analysis date in form yyyy-mm-dd_hh-mm-ss>”.

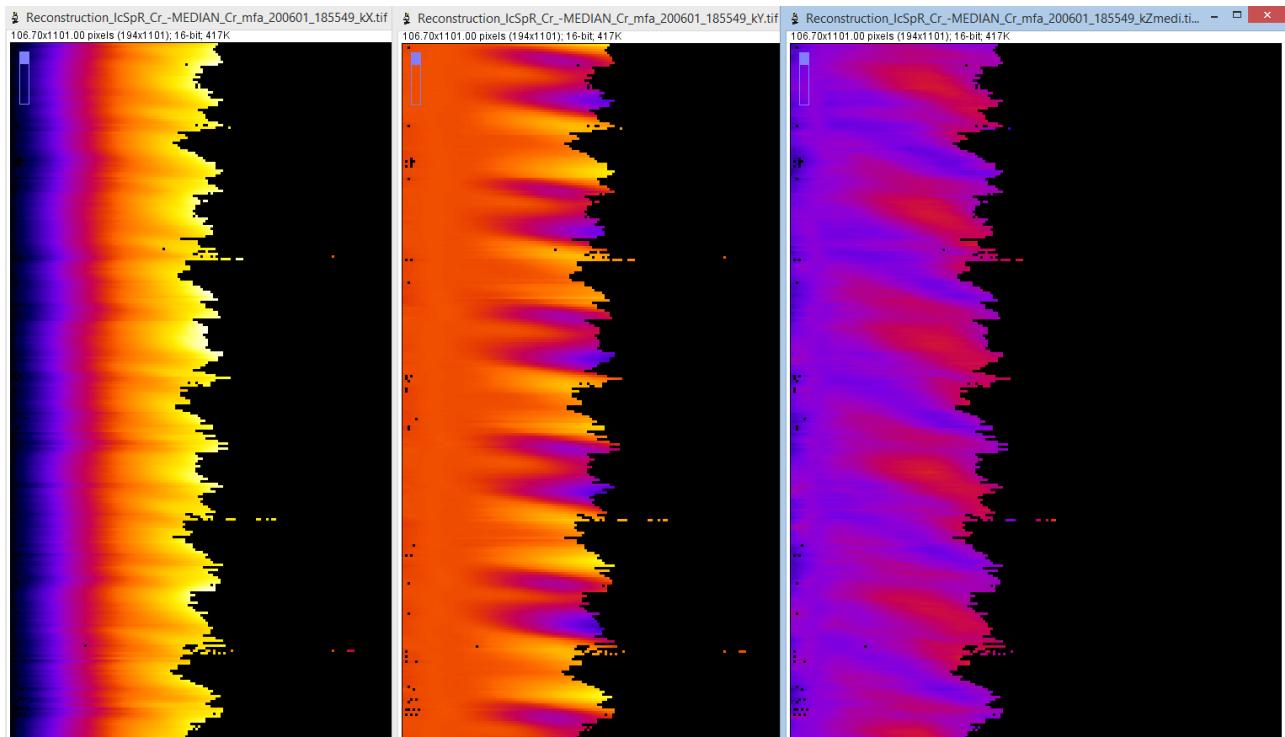


- Open the directory.

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



- The files ending with kX, kY, kZmedi contain kymographs for the x, y, and z position relative to the orientation vector (head-midpiece axis).



- To decipher the intensity code of X, Y, and Z position and convert it to the positions in μm , open the corresponding txt files:

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

The figure shows three separate Notepad windows, each displaying the same text content. The text is a header for a TIF file and includes the following information:

```

Reconstruction_IcSpR_Cr_-MEDIAN_Cr_mfa_200601_185549_kX_info - Notepad
File Edit Format View Help
Image information for image: \Reconstruction_IcSpR_Cr_-MEDIAN_Cr_mfa_200601_185549_kX.tif
dimension minimum maximum
x axis: 2D arc length 0 194 calibration [um/px]: 0,550000
y axis: time [frame] 0 1100
gray value (1.0-65534.0): oriented X position -50,000000 130,000000
code#-50.0-130.0+1.81818181818181
Ln 1, Col 1

Reconstruction_IcSpR_Cr_-MEDIAN_Cr_mfa_200601_185549_kY_info - Notepad
File Edit Format View Help
Image information for image: \Reconstruction_IcSpR_Cr_-MEDIAN_Cr_mfa_200601_185549_kY.tif
dimension minimum maximum
x axis: 2D arc length 0 194 calibration [um/px]: 0,550000
y axis: time [frame] 0 1100
gray value (1.0-65534.0): oriented Y position -130,000000 130,000000
code#-130.0-130.0+1.818181818181
Ln 1, Col 1

Reconstruction_IcSpR_Cr_-MEDIAN_Cr_mfa_200601_185549_kZmedi_info - Notepad
File Edit Format View Help
Image information for image: \Reconstruction_IcSpR_Cr_-MEDIAN_Cr_mfa_200601_185549_kZmedi.tif
dimension minimum maximum
x axis: 2D arc length 0 194 calibration [um/px]: 0,550000
y axis: time [frame] 0 1100
gray value (1.0-65534.0): oriented Z position -10,000000 100,000000
code#-10.0-100.0+1.818181818181
Ln 1, Col 1

```

- The files coordX, coordY, coordZ contain the determined X,Y, and Z coordinates of the flagellum, respectively, in μm . **New:** They can be used for further plotting, e.g. using the Matlab scripts at (<https://github.com/hansenjn/MultifocalImaging-AnalysisToolbox/tree/master/Matlab%20scripts>).

The figure shows three separate Notepad windows, each displaying a table of data. The tables have columns for time and arc length (micron). The data is as follows:

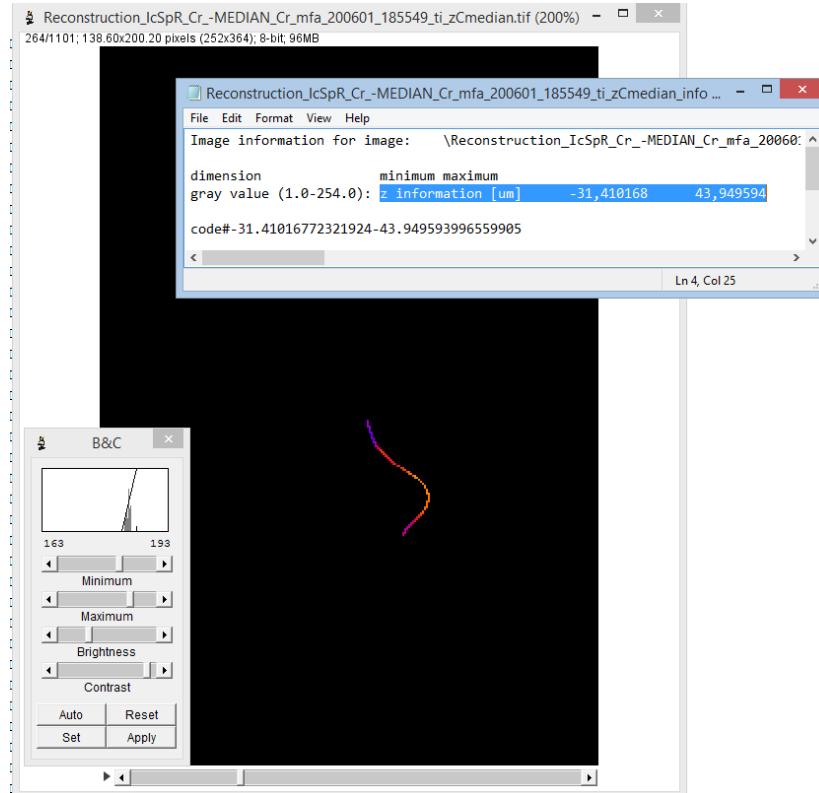
time	arc length (micron)
0,000	0,550 1,100 1,650 2,200 2,750 3,300 3,850 4,400 4,950 5,500
81,950	82,500 83,050 83,600 84,150 84,700 85,250 85,800 86,350 86,900 87,450 88,000
0	84,438268 84,648073 84,867142 85,120030 85,370844 85,61496
1	84,909245 84,909245 85,053798 85,131111 85,289575
2	85,024572 85,056373 85,276860 85,466547 85,725529 85,93075
3	85,106702 85,106702 85,290943 85,534259 85,718696 85,91610
4	85,162255 85,258931 85,357562 85,514644 85,725322 85,86286

time	arc length (micron)
0,000	0,550 1,100 1,650 2,200 2,750 3,300 3,850 4,400 4,950 5,500
81,950	82,500 83,050 83,600 84,150 84,700 85,250 85,800 86,350 86,900 87,450 88,000
0	130,618847 130,832009 131,076026 131,373972 131,685825 132,0029
1	129,590755 129,590755 130,100810 130,457573 130,795696
2	129,475428 129,537462 129,969071 130,333532 130,820015 131,2005
3	129,393298 129,393298 129,766028 130,255932 130,623938 131,0216
4	129,091421 129,310348 129,534136 129,884560 130,359524 130,6724

time	arc length (micron)
0,000	0,550 1,100 1,650 2,200 2,750 3,300 3,850 4,400 4,950 5,500
81,950	82,500 83,050 83,600 84,150 84,700 85,250 85,800 86,350 86,900 87,450 88,000
0	15,592916 15,951115 16,325725 16,745698 17,187640 17,53307
1	16,316280 15,562050 16,350572 16,708213 17,095815
2	16,316280 16,094436 16,552868 16,755106 17,088753 17,32242
3	16,316280 16,008056 16,470973 16,749807 17,080488 17,26838
4	15,187864 15,509231 16,053902 16,518599 16,893623 17,25596

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

- The file with ending “_ti_zCmedian.tif” shows the flagellar track in a stack, where the z information is intensity coded.



- SpermQ-MF determines many more parameters, analogously to SpermQ, such as the curvature angle in 2D and 3D (kcA2D, kcA3D), the curvature in 2D and 3D (kCurv2D, kCurv3D), beat frequencies (e.g. based the curvature, Curv2Df.txt and Curv3Df.txt). See the [SpermQ Publication](#) for description of these parameters.
- The summary file on the desktop (e.g. called [SpermQ-MF_Summary_200601_201105](#)) summarizes the frequency results for different parameters by thirds of the flagellum.

```
Summary results for processing of the following images:
image ID      directory      name
1      U:\Reconstruction\1-250      Reconstruction_IcSpR_Cr_-MEDIAN_Cr_T1-250_Cr.tif
#####
Average Found 1st-Peak frequencies for arc-length oriented points (X)
image ID      1st third of flagellum 2nd third of flagellum 3rd third of flagellum
1      10,000000      10,000000      10,000000
Average Found 2nd-Peak frequencies for arc-length oriented points (X)
image ID      1st third of flagellum 2nd third of flagellum 3rd third of flagellum
1      23,714286      26,769231      32,615385
Average Found COM frequencies for arc-length oriented points (X)
image ID      1st third of flagellum 2nd third of flagellum 3rd third of flagellum
1      42,530682      58,022176      56,575304
Average Found 1st-Peak frequencies for arc-length oriented points (Y)
image ID      1st third of flagellum 2nd third of flagellum 3rd third of flagellum
1      14,142857      17,692308      10,000000
Average Found 2nd-Peak frequencies for arc-length oriented points (Y)
image ID      1st third of flagellum 2nd third of flagellum 3rd third of flagellum
1      20,000000      21,076923      28,000000
Average Found COM frequencies for arc-length oriented points (Y)
image ID      1st third of flagellum 2nd third of flagellum 3rd third of flagellum
1      66,415461      60,381309      58,595818
Average Found 1st-Peak frequencies for arc-length oriented points (Z)
image ID      1st third of flagellum 2nd third of flagellum 3rd third of flagellum
1      12,307692      11,666667      10,000000
Average Found 2nd-Peak frequencies for arc-length oriented points (Z)
image ID      1st third of flagellum 2nd third of flagellum 3rd third of flagellum
1      23,692308      19,333333      25,384615
```