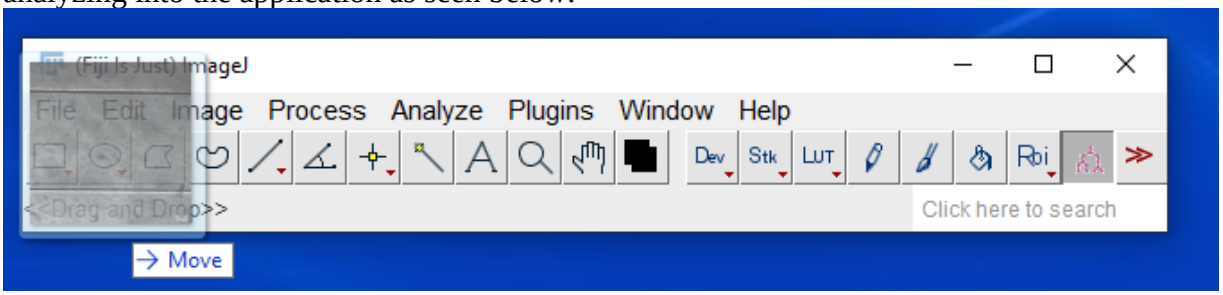
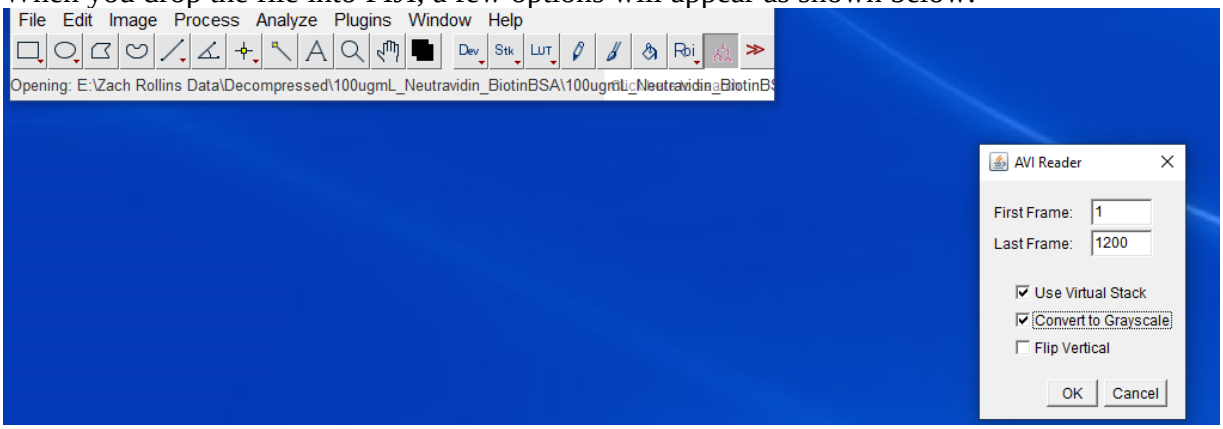
Step 1: Launching TrackMate

TrackMate is a plug-in to FIJI, which is a version of ImageJ. To use TrackMate, you must first locate the FIJI application and launch it.

Once FIJI is launched, drag and drop the file you are interested in (image stack, video, etc.) analyzing into the application as seen below.

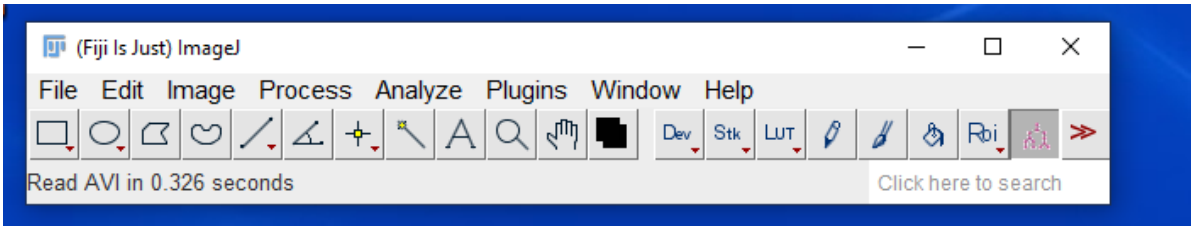


When you drop the file into FIJI, a few options will appear as shown below.



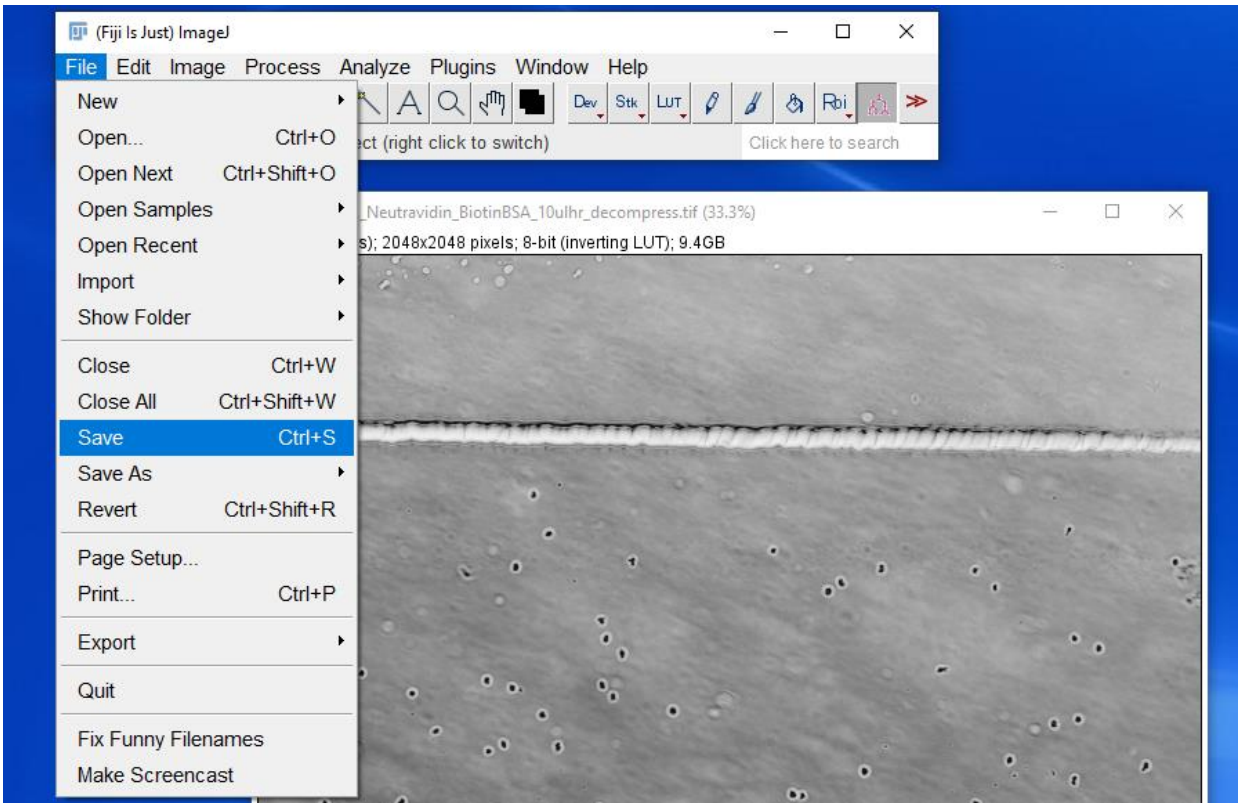
To use TrackMate on the video imported into FIJI, the video must be converted to greyscale. Make sure to click “Convert to Greyscale” to do this. An added effect is that converting files to greyscale reduces the size of the file.

After you have chosen your settings and pressed OK, the below status will appear in the area below the selectable buttons as shown below.



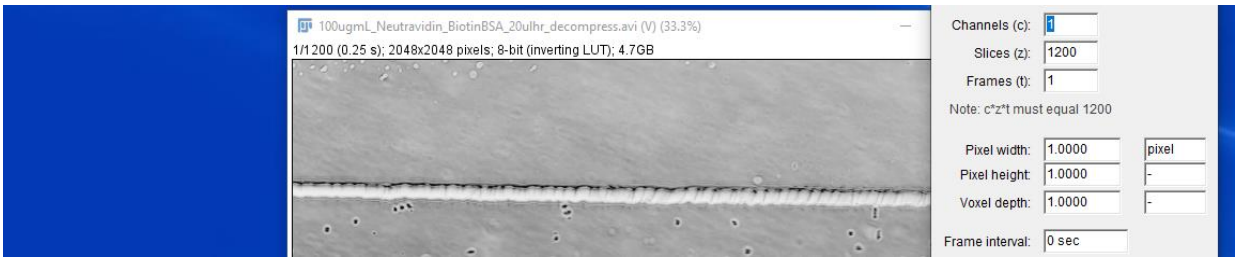
Be patient as FIJI may not immediately provide a status bar for reading the videos. Do NOT try to import the video again as this will only open two versions of the same video and double the import time.

Step 2: Saving as a TIF (Optional)
As you will find out, loading AVI files into FIJI requires the application to convert this format to a TIF file. Converting from AVI to TIF every time you want to review a video or analyze with TrackMate takes a very long time so an upfront time investment of saving a TIF file version of your video is well worth it. This can be done through the File menu or by clicking Ctrl+S as seen below.



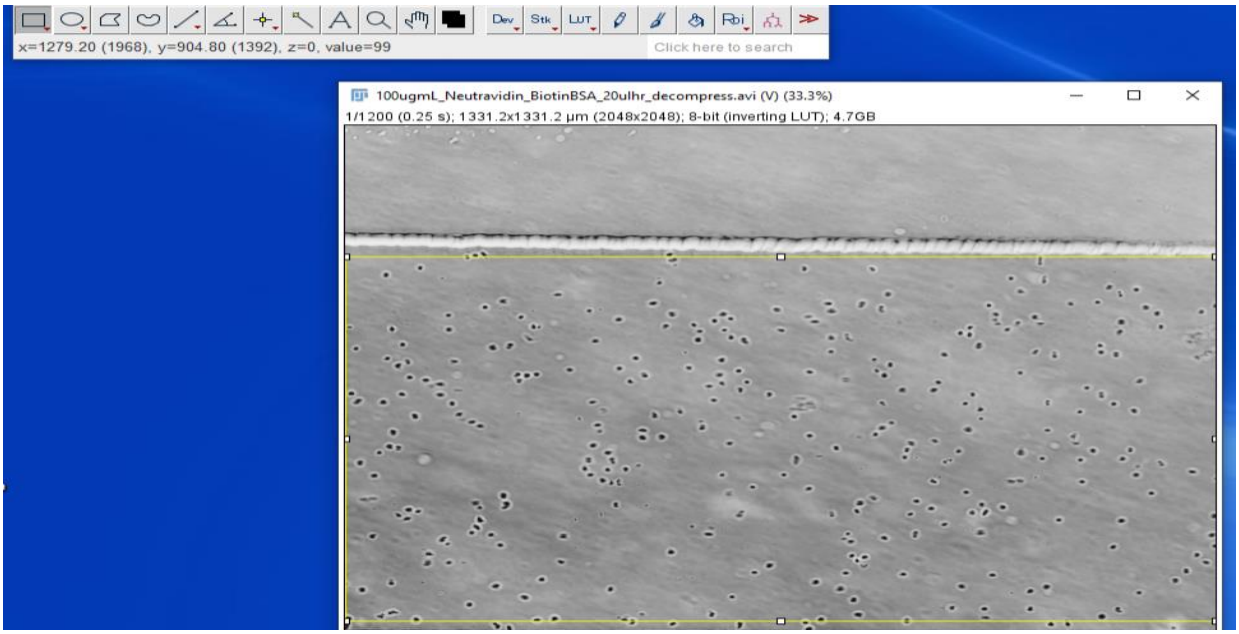
Step 3: Preparing video for TrackMate

We must ensure that the metadata of the video is correct. When importing into FIJI, the software likes to convert all the frames into a z-stack. This can be corrected by swapping the frames and slices fields as seen below.



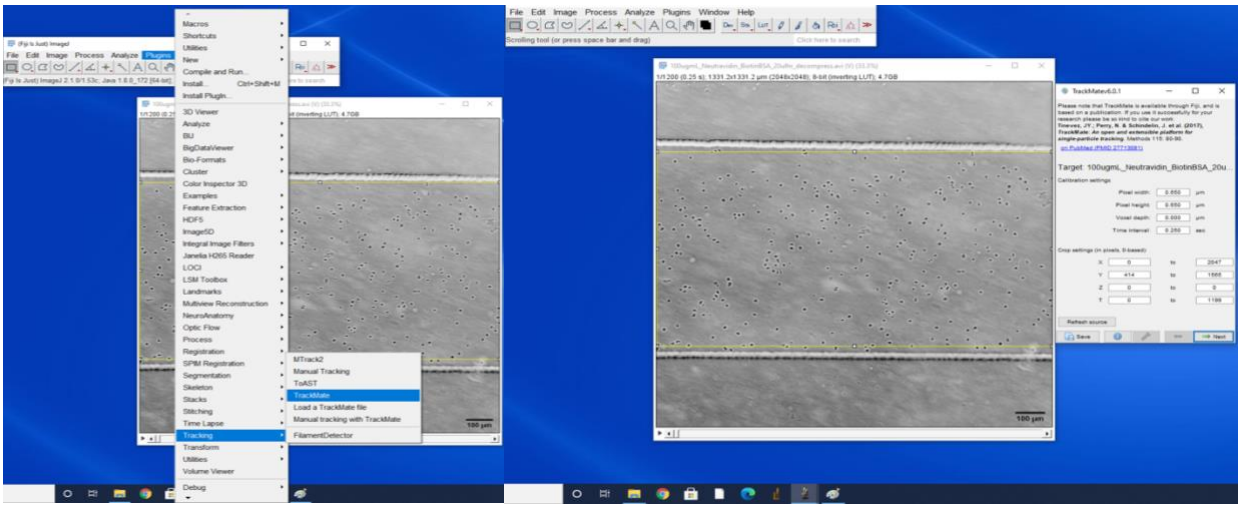
Another important area to check is the pixel dimensions. As we are using the 10x magnification, on our microscope 1 pixel = 0.65 um. Ensure that you replace the pixel width and height with 0.65 um (or your microscope specifications). As we are not concerned with, the vowel depth can be set to 0. Finally, the frame interval needs to equal the inverse of FPS.

To reduce processing time, we restrict the area over which TrackMate must scan by using the rectangle tool. Simply click and drag to surround the area of interest with a yellow box.



Step 4: Launching TrackMate

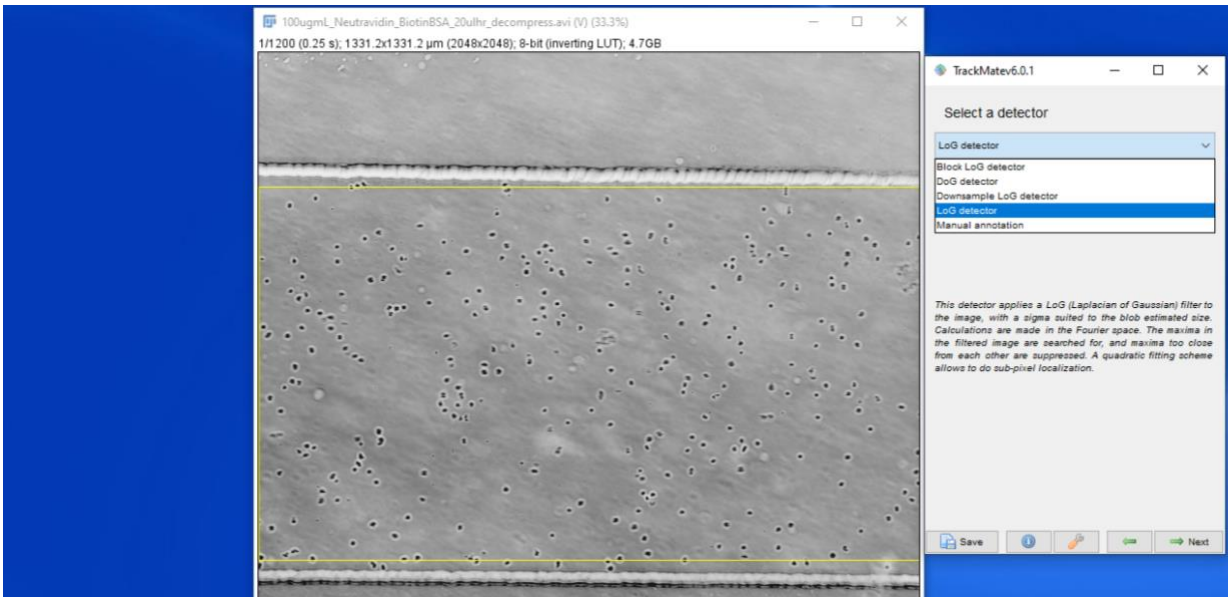
To launch TrackMate, you can either use the shortcut g or go to Plugins -> Tracking -> TrackMate. Once TrackMate is launched, a window will appear with the pixel definitions and frame intervals again. Verify that this information is correct with what you entered in the metadata. If it is not correct, close TrackMate and adjust the metadata.



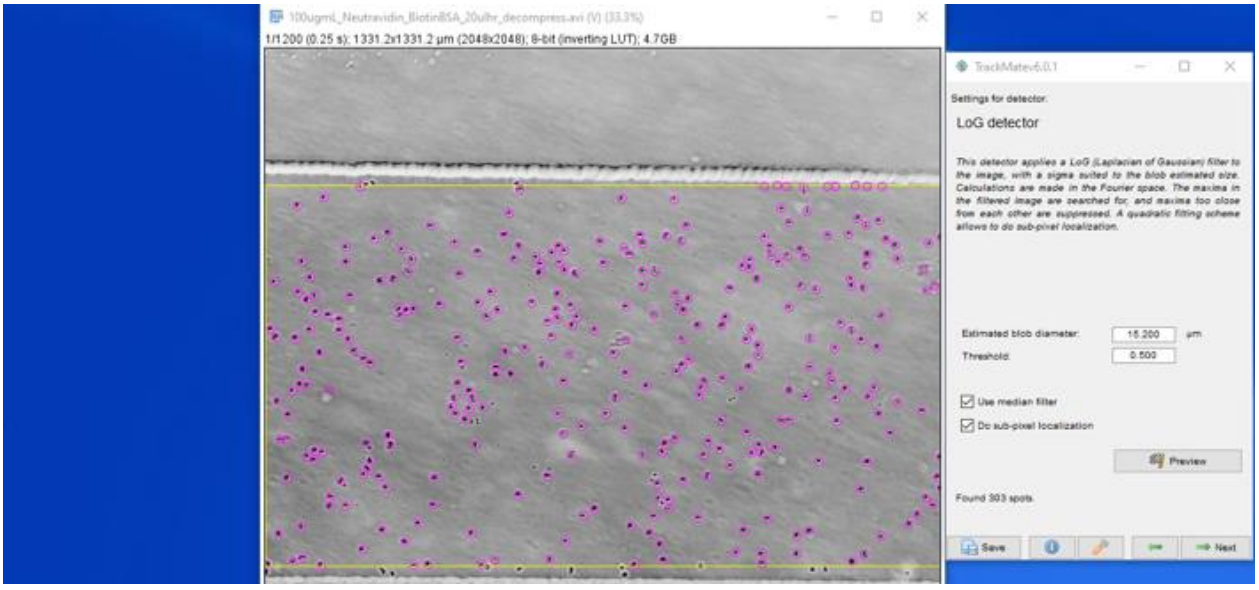
The crop settings will correspond to your selection window that you created using the rectangle tool. Ensure that this area is what you want before moving forward. Once you are satisfied with the settings, press the next button.

Step 5: Detecting spots

To detect the cells/sphere, we must choose a detector. While each detector is used for different reasons, the LoG detector is sufficient for our purposes due to the size of our cells (having a diameter of 15.2 um). Investigate additional spot detectors at your discretion.

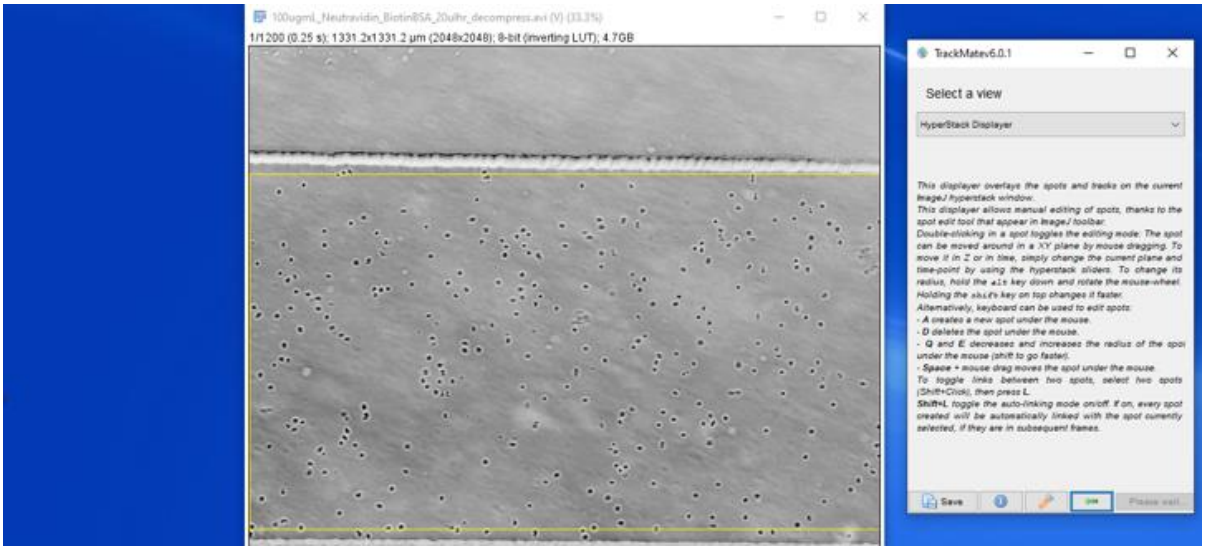


Pressing Next will take you to the next screen which will prompt you to input an estimated blob diameter (our cells) and a threshold value. For the blob diameter, we input 15.2 um and for threshold (as measured at 40X magnification), we have found that 0.300 to 0.500 generally works best. After inputting this information, check the two boxes below and then press preview. After some processing time, the following will appear.

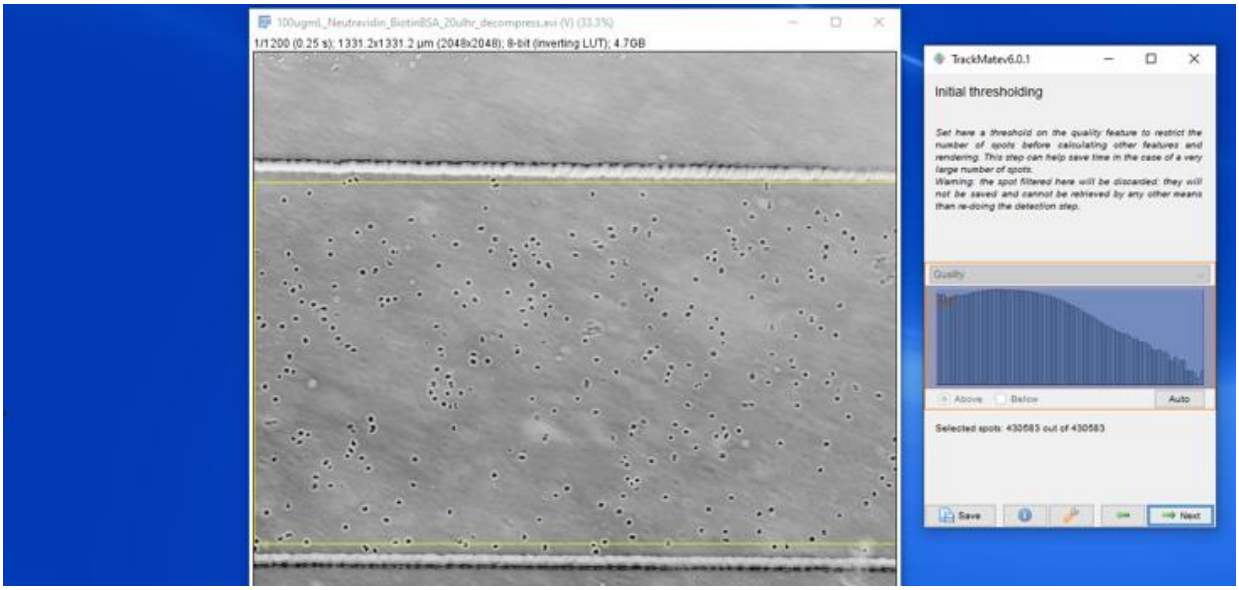


Each of the violet circles represents a “cell/sphere” that TrackMate has identified. Make sure to look carefully to ensure that most of the “cells/spheres” actually correspond. Additionally, the

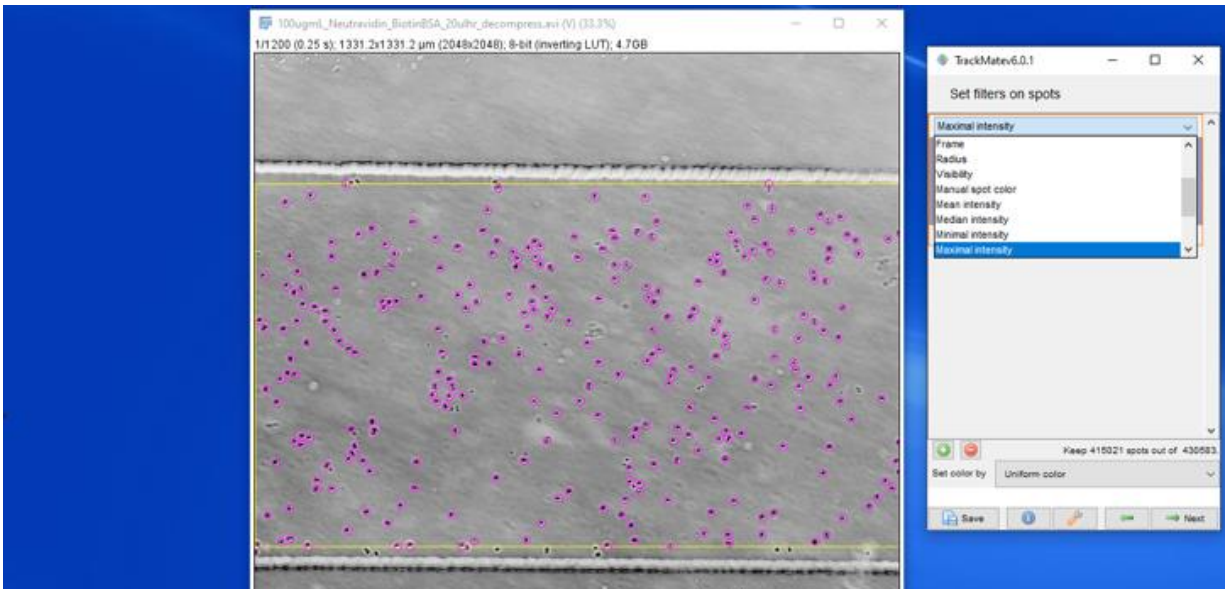
preview can be viewed at multiple frames. Ensure that the vast majority of “cells/spheres” are correctly identified, you may proceed to the next screen. This screen provides two different view options but we stick with the HyperStack Displayer.



Pressing next will allow you to do some spot filtering on the spots that TrackMate has identified as potential cells. TrackMate will ask you to apply a general “Quality” filter but since documentation on the “Quality” calculation is not sufficient, we avoid it.



Pressing next will let you to manually filter based on various categories. While there are many different filters to use, we have found that the maximal intensity filter works best to differentiate true cells from “ghosts”. Apply additional filters at your discretion.

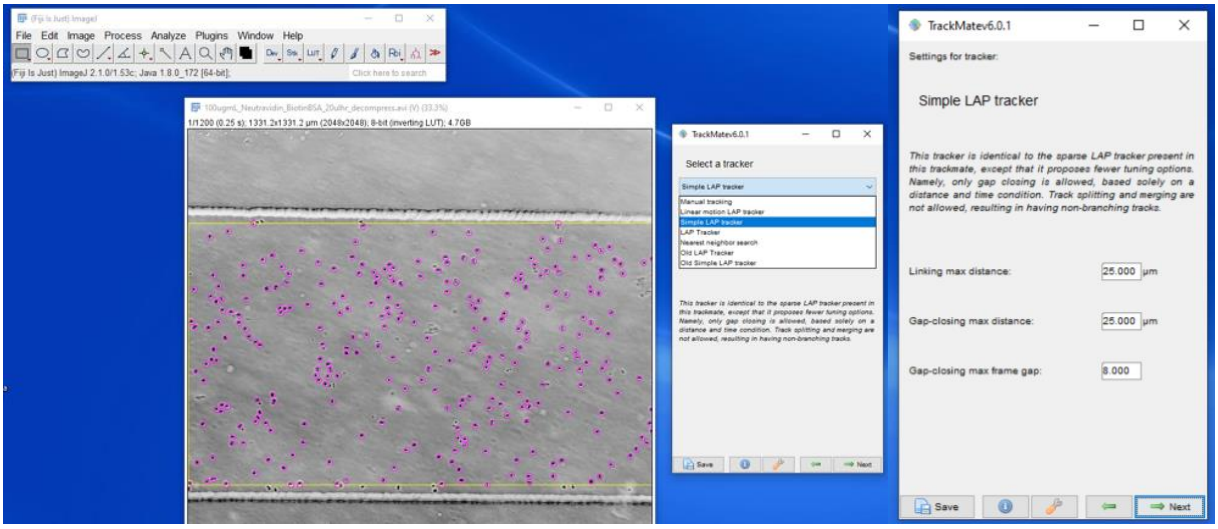


While using this filter isn't an exact science, the best strategy is to find a few frames with ghosts present and to adjust the filter up until those ghosts disappear. You can then scan the frames to make sure that this maximal intensity does not ignore any true cells. Once you are satisfied with the spot detection, you may proceed to the next step.

Step 6: Linking spots into tracks

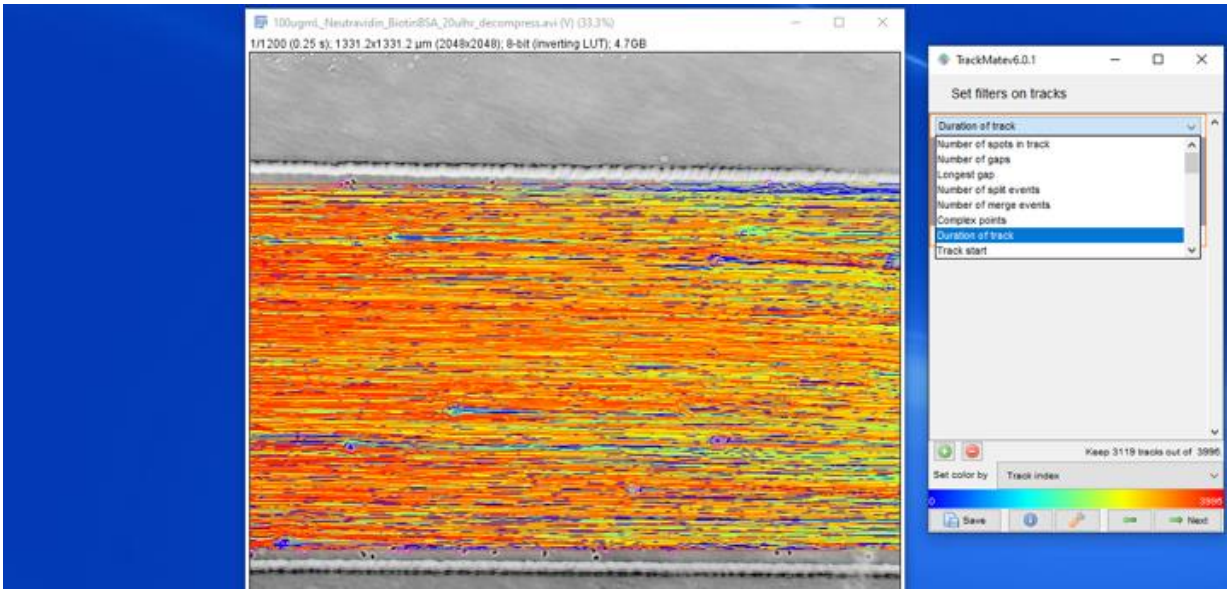
To link the spots into tracks, you must choose a tracer. We find that the simple LAP tracer is sufficient for the parallel plate flow chamber. Apply alternative tracers at your discretion. Moving onto the next window produces the parameters that are required for the tracker to narrow down the range over which it will link spots in adjacent frames. We have done some work to optimize for lower flow rates as you can see below. These will change based on flow chamber dimensions, cell/sphere diameter, etc. and need to be recalibrated with an alternate experimental setup.

Flow Rate	Max. Linking Distance	Max. Gap Distance	Max. Gap Length
5 uL/hr	15 um	15 um	2 frames
10 uL/hr	15 um	15 um	20 frames
20 uL/hr	20 um	30 um	8 frames



Step 7: Filtering tracks

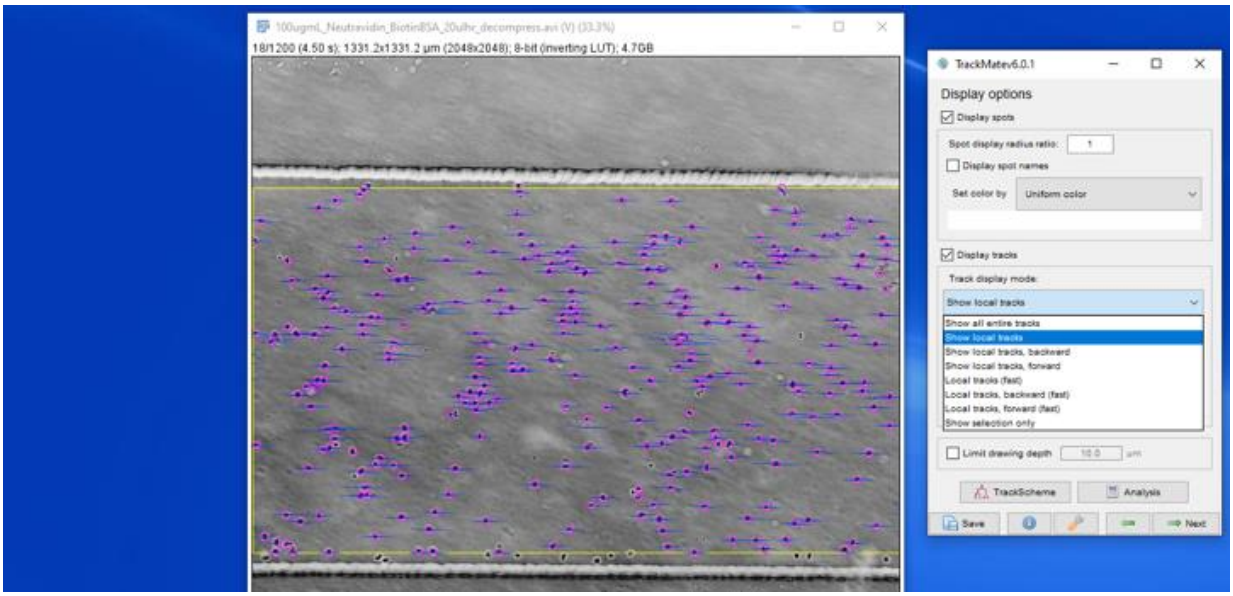
Here we filter out tracks that are too short in an effort to remove the effects of tracks ghost spots that were missed during spot filtering or tracks that are too short. A good rule of thumb here is to limit acceptable tracks to those with a track duration longer than the theoretical time for cells/spheres to cross the field of view (i.e., field of view distance / theoretical velocity).



Step 8: Tracking quality verification

Once you have filtered your tracks, it is time to check how effective your filtering strategies. This can be done on the next screen under the Display options. Under track display mode, select show local tracks or local tracks (fast). This will show the individual tracks projected forward and backward in time for each cell tracked by the program. Make sure that TrackMate is tracking the vast majority of cells and that the tracks seem to be independent from each other. The

accuracy of the tracks is crucial for calculations in RLNEK. Tracks of multiple cells should not merge and tracks should not jump around. If this occurs, you will most likely have to go back and change the max linking distance, max gap distance, and max gap frame length. If altering these does not fix the problem, you may have to go farther back into the spot detection parameters.



Step 9: Exporting results and saving progress

Once you are satisfied with TrackMates detection, you can export the data by pressing the Analysis button in the bottom right of the picture above. This will generate .csv files that will provide you with various data points pertaining to each track and spot. These .csv files can be imported into RLNEK for the calculation of relevant kinetic parameters (e.g, k_{off}, N_b/N_T, k_+, and k_{in}).