**Last updated: 11 September 2023 LS**

Before starting: make three new folders:

* Folder for the macro output, called TrackMate\_Results
* Folder for the movies you have already analyzed, called Analyzed\_movies. After following the steps below, move the movie you just finished looking at into this folder.
* Folder for low quality/out of focus cells, called Exclude. If a movie looks bad when you check it in FIJI in step 1, close out of the file and move it to this folder.

1. Open movie in FIJI by drag/drop. Inspect movie. If it looks bad/mostly out of focus, close the image and move it to the “Exclude” folder.
2. Open macro part1-TRACKMATE-openimage-adjust-invert.ijm by drag/drop into FIJI
   1. YOU WILL LIKELY NEED TO EDIT MACRO BASED ON YOUR DATA AND DIRECTORY STRUCTURE!
   2. Change outputDir in line 1 to correspond with the folder you want the macro output to be saved in. I recommend you make a new folder for this – mine was called “TrackMate\_Results”
   3. I also recommend you make a folder called “Analyzed\_movies” and “Exclude”. Then, as you run through this macro, move the analyzed movies into this folder manually.
   4. Check number of channels in your movie. Identify which channel has peroxisomes/puncta. Edit macro on lines 18-19 and 31-32 to select which channel you want to analyze.
3. Click run on the macro. TWO user prompts will come up.
   1. Graphical user interface, application

      Description automatically generatedFirst, you will be asked to rotate the image – only need to rotate the image if the cell is positioned diagonally AND there is a second cell in the field of view.
      1. TO ROTATE: Use Image/Transform/Rotate… or hotkey u on Liv’s computer.
      2. After the popup, change the settings to what is shown on the right. Yellow settings should always be the same. Green will vary.
      3. Then adjust the rotation angle (green) until the hyphae is either perfectly vertical or horizontal and select “OK”
   2. Once image has been rotated (or if you think it does not need to be rotated) click “OK”. The macro will save your rotated image in the Results folder.
   3. NEXT – the macro will ask you to trace the hyphae from tip inwards and press t to save the ROI. This is so we can measure the number of peroxisomes per cell length. Only measure the length of the cell that is in focus & where you see peroxisome signal. Then click “OK”. Macro will save the length and ROI of the line you drew in the results folder.
4. Table

   Description automatically generated with medium confidenceNow click on the peroxisome image (so the ROI disappears and the correct movie is selected) and run TrackMate: hotkey q on Liv’s computer, or Plugins/Tracking/TrackMate
5. Check that the calibration is in microns and seconds. Example right. Click Next.
6. **Graphical user interface

   Description automatically generated with medium confidenceSelect detector**: DoG or LoG detector. Click Next.
   1. Est. object diameter: 0.45 micron
   2. Uncheck Pre-process with median
   3. Check Sub-pixel localization
   4. Quality threshold will vary; usually between 0.8 and 2 for DoG, 3 to 5 for LoG. Click Preview to check the detection on your movie.
   5. You can click on the histogram to alter the quality threshhold. Ideally, the threshhold should be close to the bell curve, but not including too much of the tail. See right for example.
   6. Chart

      Description automatically generatedClick Next.
7. I usually skip **Initial thresholding**, unless the tracking looks bad later. So, for the first attempt at tracking for each video, just click “next” – make sure you are selecting all the spots by checking the numbers at the bottom – example right, with yellow. Click Next.

**Graphical user interface

Description automatically generated**

1. **Set filters on spots** – this is where you can “crop” the image to select the spots that are within only the one cell of interest. This is very useful if you have more than one cell in the crop! Use the green plus button to add filters for X or Y position. Check that the spots of interest, in purple on your movie, are the correct ones you want to analyze. See right for example of how I was able to “Crop” this movie to select only the spots in the bottom right quadrant.
2. **Graphical user interface, text

   Description automatically generatedSelect tracker:** LAP Tracker
3. Only use Frame to frame linking and Track segment gap closing. UNCHECK Track segment splitting and Track segment merging.
4. Usually the settings shown right are good enough. I try to keep the yellow values the same across all movies. But sometimes the max distance between frames (top yellow value) is better if its set to between 1.2 – 1.7 micron. I have never had to reduce it below 1.0.
5. The Feature penalties for frame to frame linking can help with linking long tracks accurately. A higher penalty score (in green) means the software will consider this feature more in linking. For example, shown right, frame-to-frame linking considers the mean intensity of two spots as more important than similarity in spot quality. I fiddle with these values – ranging 0.0 to 2.0, in 0.1 increments usually, to improve tracking on the next step.
6. Click Next. Check that the tracks make sense.
7. Chart, scatter chart

   Description automatically generatedTWO MOST COMMON TRACKING ERRORS:
   1. TRACKS ARE INCORRECTLY LINKED TOGETHER. Example shown right. Peroxisomes don’t usually move horizontally in hyphae, they usually move along the length of the hyphae. I would fiddle with the max distance and feature penalties on the previous screen until this is mostly resolved!
   2. LONG TRACK IS BROKEN INTO SMALLER PIECES. Example shown right. Again, fiddle with settings until you think the tracking represents what you see with your eyes!
8. Once you think the tracking looks accurate, click Next until you get to this screen. Graphical user interface

   Description automatically generated
9. Click the yellow button (above) to save the TrackMate .xml file – this will record all your tracking settings. Then click Resume and click the Green button (above) to download the csv file with the output data.
10. Graphical user interface, text, application

    Description automatically generatedA picture containing graphical user interface

    Description automatically generatedNext, click on the Tracks tab (green) to access the data for all tracks. Then click the blue label “Export to CSV” and save it in your output folder.
11. This save window will use the same filename as the last file you saved. So make sure to change the file name to match the movie you just analyzed, and add a suffix like “\_trackresults” – example shown right.
12. Graphical user interface, text, application

    Description automatically generatedClose all the windows. Move the image you just analyzed to the Analyzed\_movies folder and restart at step 1 for the next movie.

**Compiling the data in R:**

1. Once you have analyzed all the movies, open part2-compile\_trackmate\_output\_files.R in Rstudio. You will need to run this script LINE BY LINE and potentially edit some of the lines to get it to run smoothly!
2. Begin by setting your working directory to the TrackMate\_Results folder with your csv file outputs. In the script, line 2 should give you a popup, but if that line doesn’t work (e.g. on Mac OS) then set the WD however works best.
3. Hopefully the script gives no errors! Line 141 is for exporting data – file will be saved one directory level up, filename Summary\_trackmate\_data.csv
4. There is some code from lines 148 onwards to make graphs in R, feel free to edit or skip and use plotting software of your choice! Without a super consistent file naming structure, it is hard to plot stuff in R…