**Data Acquisition**

1. Set up a new folder in your Data folder (for example named “16.08.20”) and copy the protocol you want to use into it. Also copy the metamorph journal you will use into it, for example Loop\_3Color\_Laser.JNL
2. Make sure the Objective Correction Collar is in the right position and mount the objective plus temperature control
3. Boot up all microscope devices, including the definite focus
4. Make sure the temperature of the stage is at equilibrium at the desired temperature
5. Put your sample onto the stage (channel-to-be-imaged should be sealed)
6. Focus the sample such that you are focused in both the rhodamine and the GFP channel
7. Find a good TIRF angle
8. Set the Lamp power to 100%, the green laser to around 30 mW with 300ms exposure time (make sure you use the full dynamic range). Pre-gain should be at maximum. EMCCD at 200.
9. Focus such that both the rhodamine and the GFP channel are in focus. Do NOT manually start the definite focus (the macro will handle it)
10. Open the metamorph journal and run it. Provide the input which the journal asks for, such as the channel number
11. Afterward the movie has finished, move the newly created stacks from the local PC into your data folder

**Data Preparation**

1. Create a subfolder in which the stack is located, named after the channel number of the respective stack (for example 16.08.20\1)
2. Save all files associated with that channel in that subfolder
3. Open MásFIESTA
4. Open Stack Special -> Separate Files for Each channel -> Ch1: Rhodamine, Ch2: GFP, Ch3: Alexa 647 (if GFP available, otherwise Alexa 647 into Ch2)
5. Load the Config (I used \\bcube-files\Data\Jochen\config.mat)
6. Track seeds (blue) and extensions (red) with appropriate threshold
7. Clean the tracks crudely
   1. Merge filaments
   2. Delete obviously wrongly tracked frames and track them again (manually or with “Track missing frames”
   3. Make sure the start position of the filament is where the plus end is
   4. Do not care about individual frames too much at this point! Save your data from time to time
8. Save the filaments you just cleaned in a new file
9. Track Molecules in all channels and clean their tracks
10. Save the Molecules in a file named “Molecules”
11. Select all Molecules and set them as Drift control
12. Save the Drift in a file named “Drift”
13. Drift correct all molecules
14. Make sure you have nothing selected and create an offset map
15. Save the Offset map in a file named “Offset”
16. You don’t need the molecules in your FIESTA session anymore from this point on, so you can delete them if you wish
17. Select all Filaments and rename them (right click -> unique names for selection)
18. Drift correct
19. Apply Offset map
20. Align Channels
21. Open Statistics -> Dynamic Filaments
22. Make sure you have all Filaments loaded in both the extension and the seed channel
23. Click “Match Filaments” (this will find the seed for each extension, i.e. it will add a comment to each Filament in channel 1 saying ref:<seed name> )
24. Fill in the type of your Filaments and click “apply type”
25. Click “Interpolate Tracks” (this will interpolate the positions of the seeds such that the positions become available in all frames of the stack where you tracked the extensions)
26. Right click -> Sort Filaments by channel, so that the extensions come first
27. Make sure you got every
28. Go through each data view of your MT extensions
    1. Create a drift-corrected multicolor kymograph (after having set the “correct drift” checkbox in the kymograph menu you can conveniently create these pressing “kymograph” in the data window
    2. The most useful graph now is to select “time” for the x-axis and “distance to reference” for the y-axis, the second most useful is x versus y
    3. Check whether the referenced seed name is correct
    4. Fix individual frames, especially the frames during shrinking segments (remove the drift correction temporarily to use “fit missing frames”)
    5. If you can’t fix the frames or if the extension tip is somewhere it should not be according to its type, tag the corresponding frame(s). Find a list of which tags to set clicking the “Tag Code” button in the data window of your filament.
    6. Also tag catastrophes and rescues (does not have to be the exact frame, this is later just used for verification)
    7. Save the kymograph (both tif and pdf) with the filaments name
    8. Move to the next Filament
    9. If a Filament cannot be used for analysis, you can simply write a double-dash (“--“) into its comments
29. When done, save all filaments under a new file called “x\_dynamics” (the \_dynamics signals that this is the file to be analyzed)

**Data Analysis**

1. Open the file in the Dynamic Filaments GUI (DyFiGui)
2. With the correct segmenting options loaded, go through all Filaments to check whether there are any false positives or false negatives (catastrophes/rescues)
3. If there are, go back to FIESTA and fix them by tracking anew or, if not possible, appropriate tagging
4. When done, reload the filaments into the DyFiGui and check again
5. If everything is OK, also load the link file containing all the other files you want to analyze together with the current file (for example “16.08.20\2\3\_dynamics”, “16.09.01\1\dynamics” etc)
6. Click “Save Links” and overwrite the currently loaded link file. It will now also contain your newly prepared filaments
7. You can now get and set the filament’s intensity and intensity per MAP via various methods, see chapter “Intensity”
8. Analyze your data using the DyFiGui.