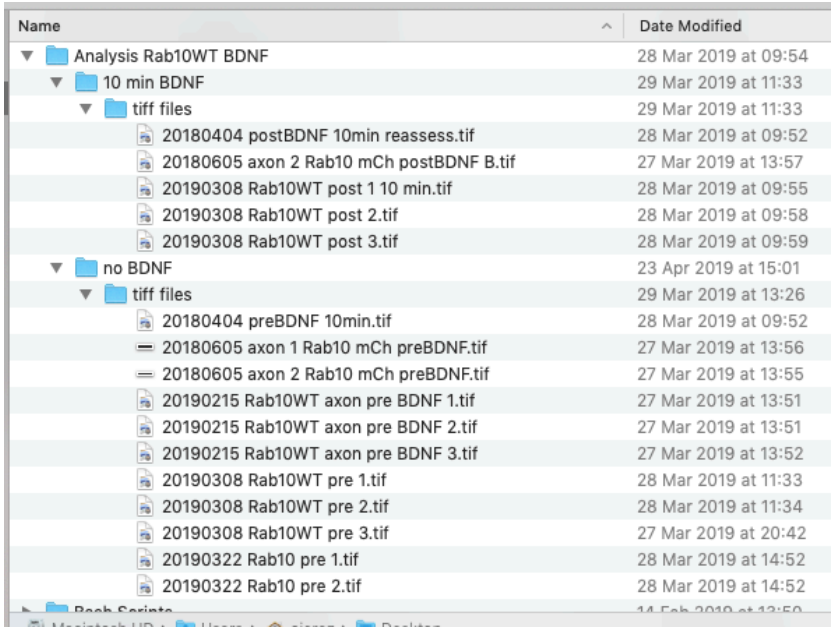
Analysing kymographs by using Kymoanalyzer v. 1.01

1. Files organisation:

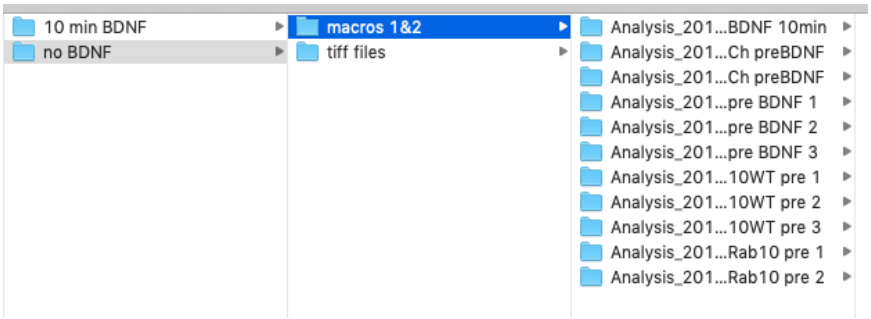
— Make one folder per experimental condition. Within that folder create one for TIFF files (folder A, in this example it is called "tiff files") and put all of them there (make sure are .tif files).



— You will create 2 other folders later on (B and C), all of them within the **condition folder**. Is not required to create them now.
— Make sure the videos are in consistent spatio-temporal scales and correct the drifting.

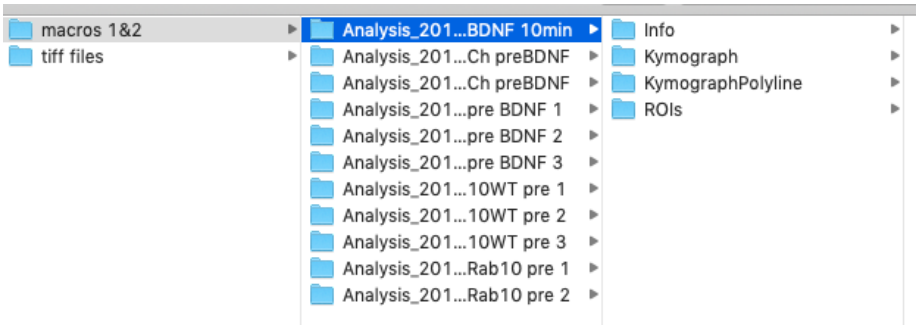
2. Run the analysis:

— **Run Macro 1**. Indicate that the filetype is .tif and select the folder containing the TIFF files (folder A).
— When you do the polyline, make sure it covers the axon at any point of the video. If necessary, correct any significant drift in advance.
— Before running Macro 2, **create the folder B (in this example is called "macros 1&2") and move the Analysis folders to this new folder**.

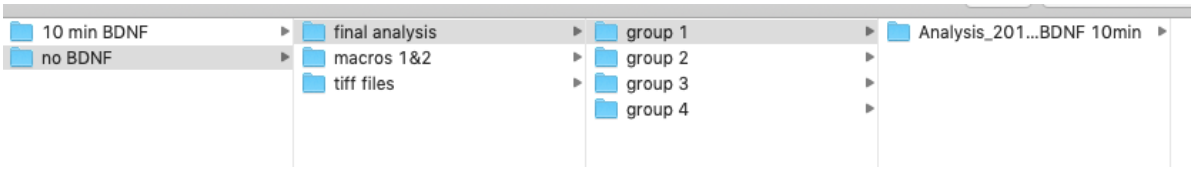


— It's a good idea to check in *Edit > Options > Colors* that the selection colour is set to **yellow** (or a distinctive colour of your preference), and in *Image > Overlay > Overlay* options set the colour of the overlay to (let's say) **cyan**, you'll use the overlay to label the tracks you have added during the process and avoid duplication.
— **Run Macro 2.** Select one Analysis folder at a time within folder B (from the ones you just moved from folder A). Trace a new track and then press *cmd+b* before accepting the track, in order to **label it cyan**. Accept the track and add the next one. Continue until there is nothing else to be tracked, and then answer NO.

— Duplicated tracks will be deleted automatically but problems can arise if horizontal or inconsistent segments are detected. There is no way to edit and save the modifications at this point, so I suggest to identify the problematic tracks, close the window, run the Macro in this folder again (all the selected tracks in the ROI manager are going to be re-loaded), delete whatever is causing the problem and trace again those tracks as normal. Then finish this step with no errors.
— Once you have applied Macro 2 to an Analysis folder the content of the folder changes, so **you can know at any point which ones you already processed**. The completed folder will have 4 sub-folders.



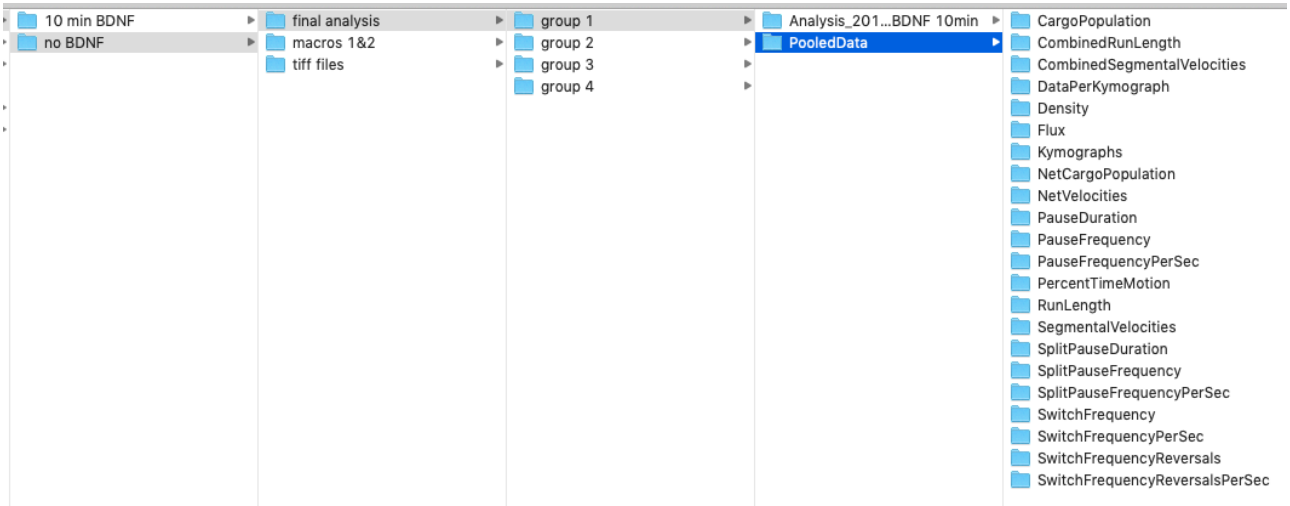
— As I said at the beginning, consistency between the scale and frame rate of the different videos is crucial to have consistent data. But if you do have certain degree of heterogeneity in resolution or frequency of the videos you still can implement the analysis, you just need to make sure you group the videos that are consistent in separated folders so you can apply Macros 3, 4, 5 and 6 separately. Before proceeding into Macro 3, **create folder C (in this example is called "final analysis")** and move or copy the content from folder B to the new folder C. Here the similar videos were grouped in subfolders.



— **Run Macro 3, 4, 5 and 6.** From the folder C (or the respective group sub-folder if you have the videos grouped). Remember putting the appropriate pixel size and frame rate for the video you are analysing, that is the data that make your results comparable.

3. Gather and analyse the data:

— Once you finish of running all the macros, you will have a folder called **"Pooled data"** where you have all the tables for that group.



— **Direction:** You will find the total number and fraction of retrograde, anterograde, reversal and immobile tracks in the folder "Cargo population". Immobile are defined as the ones that don't go >0.35µm away from the midline of the track.
— **Average velocity when moving**: You can find the average velocity of every segment (moving trajectory between pauses) grouped by direction in the folder "Segmental velocities". You can use them also to produce a **speed profile** integrating retrograde an anterograde velocities.
— **Pausing:** The proportion of time that every cargo spent in pausing (velocity < 0.2 µm/s) separated in retrograde and anterograde population.
— Pausing threshold was modified locally. In the original macros the minimum velocity for being considered a mobile segment is 0.1 µm/s. The macros has been locally modified to use the functional Multiple Kymograph, since the one used in the macro isn't included in FIJI/ImageJ anymore.
— Other analysis can be done. You can find more information and examples at: httpps://www.encalada.scripps.edu/kymoanalyzer

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