KymoAnalyzer User Manual
(Version 1.0)

This manual provides a step-by-step guideline to installing and running KymoAnalyzer. A few notes and tips on things to consider while making manual track assignments are also included.

We recommend to use Fiji *(Fiji Is Just ImageJ*), an open source processing package based on ImageJ (1), as it is pre-loaded with all the *plugins* necessary to open raw time-lapse image sequences generated by any microscope. Fiji can be downloaded at: http://fiji.sc/Fiji

I. Installation and pre-KymoAnalyzer analysis guidelines

1. Pre-install the plugin ***MultipleKymograph*** in ImageJ. This plugin can be downloaded from http://fiji.sc/Multi_Kymograph
    - Download MultipleKymograph_.class, to the ImageJ plugins folder and restart ImageJ.

2. The ***KymoAnalyzer*** package contains 6 macros. Unzip the file *KymoAnalyzer_ImageJPackage.zip,* copy the folder into your ImageJ plugins folder and restart ImageJ. The KymoAnalyzer can be downloaded as a zip file from our laboratory website: **http://www.encalada.scripps.edu/kymoanalyzer**

   After installation, the KymoAnalyzer package and its 6 macros should be visible in ImageJ, in the tab ImageJ/Plugins (Figure A).

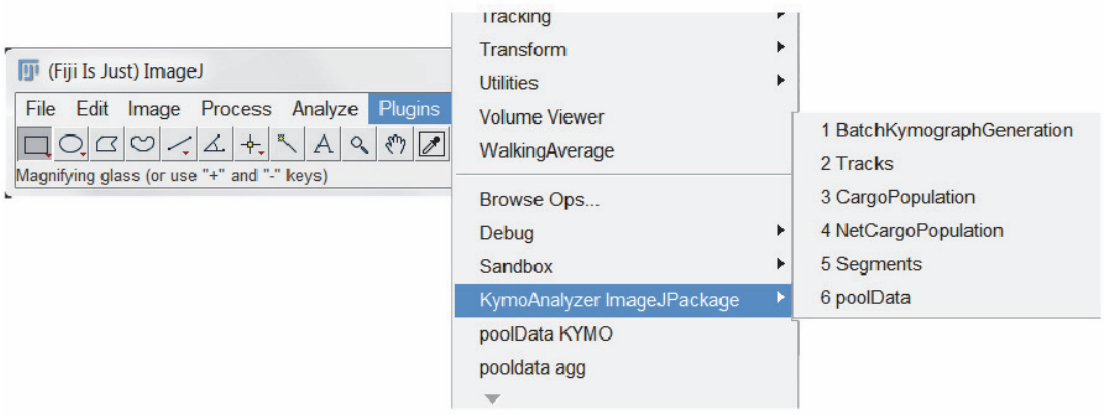


Figure A. KymoAnalyzer package can be launched from the ImageJ/Plugins tab.

3. All movies to be analyzed must have the same format, the same frame-rate and the same resolution (pixel size). Movie formats include all file type that are supported by the Bio-formats library (.nd2, .tif, and .stk).

4. In order to facilitate the analysis of the data generated by KymoAnalyzer after data pooling, we recommend that you create the following blue folders indicated in ***Figure B***, before you start the KymoAnalyzer analyses (blue folders are created by the user, while yellow folders are created by the KymoAnalyzer software):

   Create **Condition folders**: one for each of your conditions (e.g. for each genotype) to be analyzed.

   Create **Experiment folders** inside each **Condition folder**. For example, **Experiment folders** could comprise experiments run on different days for a specific condition. Place acquired movies to be analyzed for each experiment inside corresponding **Experiment folders**.

   Place only your movie files in the **Experiment folders**. KymoAnalyzer will generate new files and folders within each **Experiment folder** that will contain transport parameter analyses (yellow folders in ***Figure B)***.

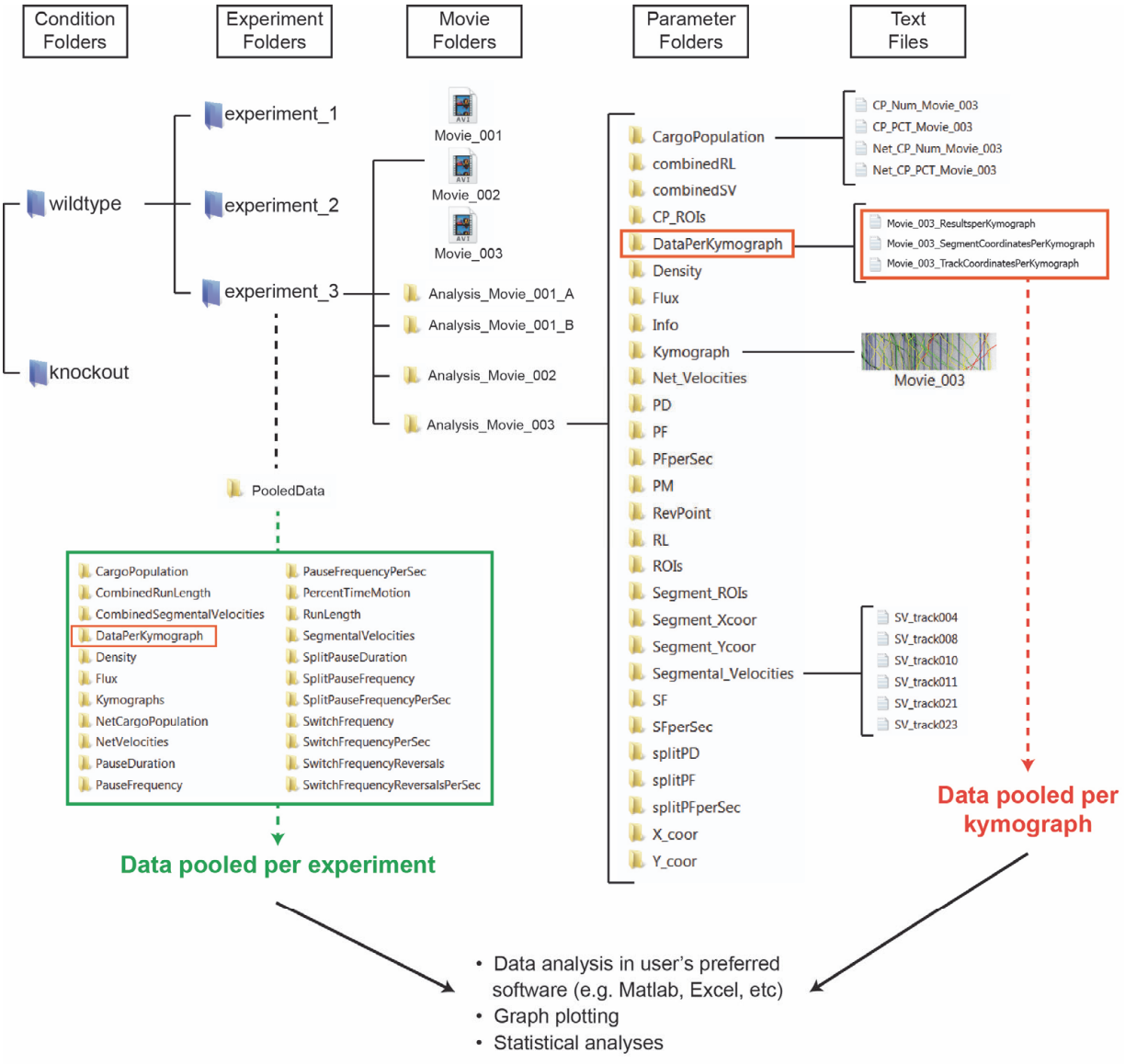


Figure B. KymoAnalyzer directory organization

Blue folders (*Condition Folders* and *Experiment Folders*), are created by the user prior to running KymoAnalyzer. Raw movies corresponding to a specific experimental condition are placed within each *Experiment Folder*. Yellow folders correspond to the system directory that is generated by the successive KymoAnalyzer macros. Within each *Experiment Folder*, a specific *Movie folder* is created for each movie. If the user decides to create multiple kymographs from different neurites present in the same movie, individual *Movie folders* are generated for each neurite and arranged alphabetically. Kymograph images and track coordinates assigned by the user are saved in specific folders within *Movie Folders*. All the calculated transport parameters are saved as text files and sorted in specific *Parameter Folders*. The analysis of individual kymographs is facilitated by the generation of spreadsheet files summarizing all the parameters, as well as the clicked coordinates of each particle (orange box). The *poolData macro* combines the data from all the movies located in an *Experiment Folder* (green box). Pooled data can be easily used to plot figures or run statistical analysis post-KymoAnalyzer analyses. See ***Materials and Methods*** and ***Supplementary file 2*** for description of transport parameters. RL: segmental run length; SV: segmental velocity; CP: cargo population; NetCP: net cargo population; ROI: region of interest; PD: pause duration; PF: pause frequency; SF: switch frequency; PM: percent time in motion.

II. Step-by-step procedure for running KymoAnalyzer

The ***KymoAnalyzer*** package contains six macros that should be run sequentially as indicated below:

1. *BatchKymographGeneration*
2. *Tracks*
3. *CargoPopulation*
4. *NetCargoPopulation*
5. *Segments*
6. *poolData*

1. BatchKymographGeneration

The ***BatchKymographGeneration*** macro guides the user in the generation of high-resolution kymographs. The user has the possibility to generate multiple kymographs from different neurites or isolated microtubules present in a single movie.

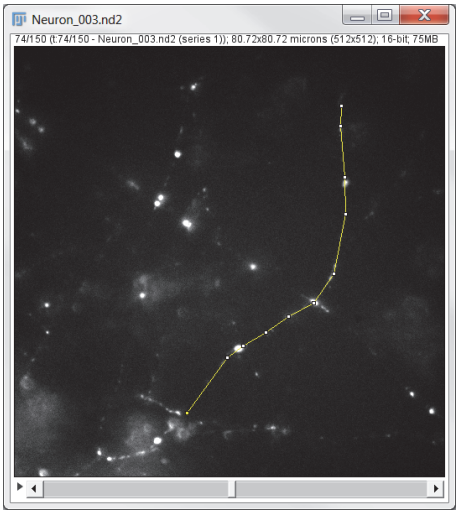
1. In ImageJ, run the ***BatchKymographGeneration*** macro by clicking on Plugins/ *KymoAnalyzer_ImageJPackage*/*BatchKymographGeneration*

2. Select the **Experiment folder** to be analyzed (**Figure B**). The folder should only contain the movies.

3. Indicate the file suffix of your movies (.nd2, .stk, .tif, …).

4. If you want to generate more than one kymograph per movie select “yes”

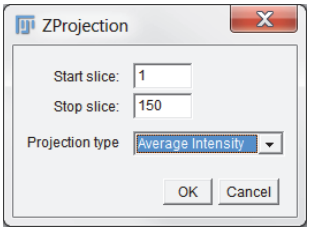
5. The movies will be opened sequentially. For each movie, draw the neurite trajectory using the polyline tool. Double click at the end of your track. Use the same directionality for each neurite. For example, start the polyline always at the cell body side and end at the synaptic side. Note that movies do not to need to be oriented, resized or cropped.



6. When done, click “OK”. The user has the possibility to draw up to 26 neurite trajectories per movie. When done with a movie, the next one will be opened automatically.

7. New folders (**Movie folders**) will be automatically generated for each kymograph. The kymographs are automatically saved inside the **Movie folders** (***Figure B***).

Comment: It is sometimes difficult to visualize the neurite from a still movie frame (especially when the cargo of interest do not present any cytosolic or plasma membrane staining). To improve the signal-to-noise ratio and detect the neurite trajectory more easily, the user can generate a time projection of the movie while using the ***BatchKymographGeneration*** macro. When asked to draw the neurite trajectory (point 5 above), use the ImageJ command “Image/Stacks/Z Project…” and select the projection type “Average Intensity” (see image below).



Then, draw the neurite trajectory using the polyline tool on the time projection image and use the command “Edit/Selection/Restore selection” (Ctrl+Shift+E in ImageJ), to copy the polyline on the Movie stack. Close the time projection image.
When done click OK, it will process to the next movie (point 6 above).

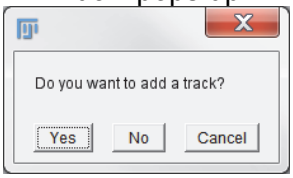
2. Tracks

The ***Tracks*** macro guides the user in the manual tracking of particle trajectories from kymograph images. Before running the Tracks macro, original/raw movies must be removed from the **Experiment folder** (they are not needed after kymograph generation and might cause errors in the subsequent analysis). Only the **Analysis folders** should be kept in the **Experiment Folder**.

1. In ImageJ, run the ***Tracks*** macro by clicking on *ImageJ/Plugins/KymoAnalyzer_ImageJPackage /Tracks*

2. Select the first **Movie folder** to be analyzed. Note that ***Tracks*** is the only macro that has to be run individually for each **Movie Folder**. All the subsequent macros can process multiple kymographs at a time.

3. A window pops up.

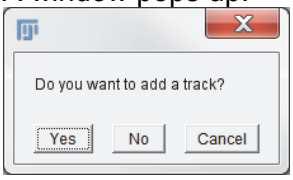


Click “Yes” to add the first track.

4. Draw the trajectory of the first track using the polyline tool. Double click at the end of your track. You can start from the top or from the bottom of the kymograph at your convenience. [You can easily set the brightness and contrast settings at any time to help you visualize the cargo trajectories] (***Figure C***).

5. Press “OK” when done.

6. A window pops up.



Click “Yes” to add a track. Repeat this process until you are have finished clicking all tracks. The user can show/hide the track lines of the previously clicked particles by checking/unchecking the “Show all” box in ROI manager window (**Figure C**).

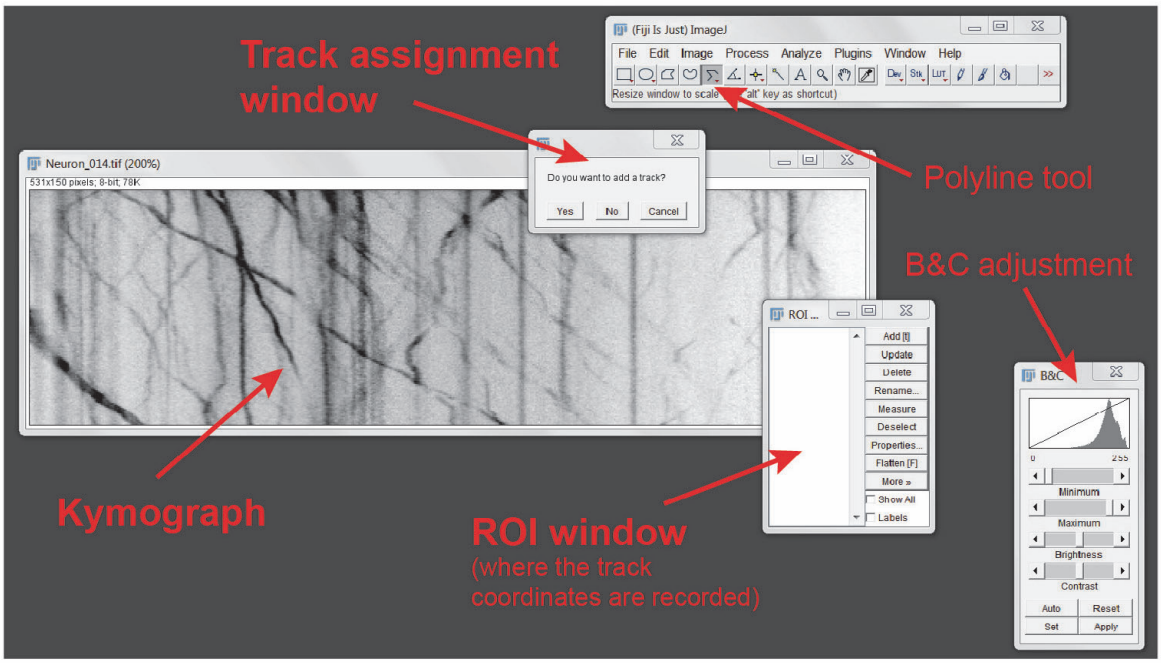


Figure C. Overview of the manual assignment of tracks using the *Tracks* macro

7. When you have finished the assignment of all tracks of a specific kymograph, click “No” in response to “Do you want to add a track?”. The software offers you the possibility to remove tracks using the ROI manager.

8. The macro checks for the presence of duplicated tracks or any horizontal lines because it would generate errors during the subsequent calculation of parameters. If the macro identifies a track with horizontal lines, this track has to be manually corrected. In macro generates a subfolder called “Info” in the **Movie folder** and saves a txt file called “Check

tracks" in addition it prints the same information in the Fiji log window. This file contains the name of the tracks that contain horizontal lines. To manually correct a track with horizontal lines, do the following:

- Rerun the macro ***Tracks* on the same movie.**
- Choose "Yes" to the question "Do you want to add a track.
- Redraw a new corrected track. Do not adjust the vertices of the track containing horizontal lines, because these changes cannot be recorded by the macro. The newly drawn track will be added as a new track to the ROI manager.
- Choose "Yes" to the question "Do you want to remove the track".
- Select the track containing horizontal lines in the ROI manager
- Press delete in the ROI manager menu.
- Press "OK" in the pop-up menu.

9. You can close the kymograph and move on by running the Tracks macro on the next **Movie folder.**

   If you need to add a track after you have closed a kymograph, you can do it by running again the Tracks macro on this specific kymograph.

   To ensure saving your data, we recommend to make a copy the entire Condition and Experiment folders at this point in your analyses.

   In addition, for the next macros to run properly, remove the original/raw movies from the **Experiment folder** after this step (they are not needed; only the ROIs will be read).

3. CargoPopulation

The ***CargoPopulation*** macro processes automatically all the kymographs contained within an **Experiment folder** and calculates Cargo population, Density, Flux and Switch frequency parameters (see ***Material and Methods*** for description of parameters).

1. Run the ***CargoPopulation*** macro by clicking on ImageJ/Plugins/ *KymoAnalyzer_ImageJPackage*/*CargoPopulation*.

2. Select the **Experiment folder** to be analyzed. A window pops up. You are prompted to enter the pixel size (µm) and frame rate (number of frames/sec) of acquisition. This step is fundamental to ensure the correct calculation of parameters.

The kymographs are processed successively and are closed at the end of processing. After this macro is run, each **Movie folder** will be populated with various sub-folders (**Parameter folders**) containing the parameters stored in text files (***Figure B***). For example numerical Cargo population parameters are stored in the file **CP_Num_Moviename.txt** located in the sub-folder **CargoPopulation**. Density parameters are stored in the file **Density_Moviename.txt** located in the sub-folder **Density,** etc.

4. NetCargoPopulation

The ***NetCargoPopulation*** macro processes automatically all the kymographs contained within an **Experiment Folder** and calculates Net cargo population and Net velocity parameters (see ***Material and Methods*** for description of the parameters).

1. Run the ***NetCargoPopulation*** macro by clicking on ImageJ/Plugins/ *KymoAnalyzer_ImageJPackage*/*NetCargoPopulation*.

2. Select the **Experiment folder** to be analyzed. A window pops up. You are prompted to enter the pixel size (µm) and frame rate (number of frames/sec) of acquisition. This step is fundamental to ensure the correct calculation of parameters.

The kymographs are processed successively and are closed at the end of processing. After this macro is run, each **Movie folder** will be populated with various sub-folders (**Parameter folders**) containing the parameters stored in text files (***Figure B***). For example, Net velocity parameters for each track are stored in the files **NV_tracknumber.txt** located in the sub-folder **Net_Velocities**.

5. Segments

The ***Segments*** macro processes automatically all the kymographs contained within an **Experiment folder**. It detects individual segments and pauses within each track trajectory and calculates Segmental velocity, Run length, Pause duration and Pause frequency parameters (see ***Material and Methods*** for description of the parameters).

1. Run the ***NetCargoPopulation*** macro by clicking on ImageJ/Plugins/ *KymoAnalyzer_ImageJPackage*/*Segments*.

2. Select the **Experiment folder** to be analyzed. A window pops up. You are prompted to enter the pixel size (µm) and frame rate (number of frames/sec) of acquisition. This step is fundamental to ensure the correct calculation of parameters.

The kymographs are processed successively and are closed at the end of processing. After this macro is run, each **Movie folder** will be populated with various sub-folders (**Parameter folders**) containing the parameters stored in text files (***Figure B***). For example Run length parameters for each track are stored in the files **RL_tracknumber.txt** located in the sub-folder **RL**.

6. poolData

The poolData macro compiles the data calculated from the macros III-V for multiple movies within a single **Experiment folder**, and stores them in a folder called ***PooledData***, as text files specific for each parameter.

1. Run the ***poolData*** macro by clicking on ImageJ/Plugins/ *KymoAnalyzer_ImageJPackage*/*poolData*.

2. Select the **Experiment folder** containing the data to be pooled. Make sure to run the ***poolData*** macro after running all the other macros. No further action from the user is necessary. Wait until the line: Command finished appears in the Fiji window. The macro

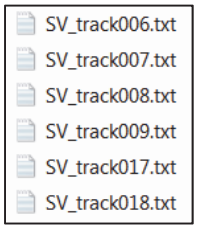
runs in the background and dependent on the amount of data might take a few minutes to be completed.

Please note that the user can reorganize the **Movie folders** before running the ***poolData* macro**. For instance, the user can decide to exclude some movies/kymographs from the final analysis by deleting the corresponding **Movie folders**, or decide to analyze subsets of movies by placing specific **Movie folders** in separate **Experiment folders** created by the user.
The purpose of the **Experiment folder** is to facilitate the pooling of the data coming from a set of movies/kymographs. Reorganizing the **Movie folders** after running macros III-V and before running ***poolData*** macro does not affect the data pooling.

III. Tips on how to access and extract the data

Calculated parameters are stored in text files. The data can be copied/pasted or directly imported into common spreadsheet, figure-plotting, or statistical software (including Excel, MATLAB, R and SPSS). The versatility of the KymoAnalyzer allows the user to perform transport analyzes at various levels, including track by track, kymograph by kymograph and whole dataset comparisons.

Access individual track data:
Data for each individual track of a particular movie/kymograph can be found in the sub-folders (**Parameter Folders**) of the **Movie Folder**.
See example below for Segmental velocity parameter. Each file contains the successive segmental velocities of each track.



In addition, coordinate list of each individual track is stored in a table, located in the folder “DataPerKymograph” of each Movie Folder.

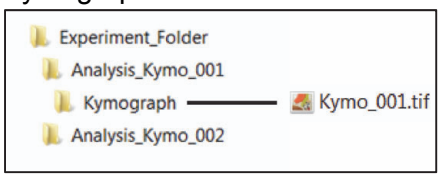
Access individual movie/kymograph data:
Data for each individual movie/kymograph can be found in the sub-folders of the **PooledData** folder.
To facilitate the comparison of individual movies/kymographs, tables summarizing all the parameters calculated from a particular movie are generated. Those files can be found in the folder “DataPerKymograph” of each **Movie Folder**, as well as in the PooledData folder (**Figure B**).

Data per experiment (set of movies):
The PooledData folder also contains files pooling all the data coming from a complete dataset of movies. To facilitate the subsequent analysis, parameters are sorted by directionality, as shown below with Net velocity parameter.

IV. Procedure to upload and analyze pre-existing kymographs

The KymoAnalyzer is compatible with kymographs that were generated beforehand or with a different software. It is possible to upload kymographs, rather than movies, and analyze them with the KymoAnalyzer. We recommend to upload kymographs in .tif format. It is also important to know the temporal and spatial resolutions of the kymographs.

The procedure to upload kymographs is at follows:
For a kymograph called “Kymo_001.tif”:
Create a movie folder with the name Analysis_Kymo_001
Create a subfolder called Kymograph within the folder Analysis_Kymo_001
Paste the Kymograph image named “Kymo_001.tif” in the folder Analysis_Kymo_001/ Kymograph



Repeat the procedure for the next kymograph. Names on the kymograph image and Movie Folder must correspond.

Procedure to trace the track lines on uploaded kymographs:
Skip *BatchKymographGeneration* macro and run directly *Tracks* macro.
All the subsequent steps of analysis are unchanged.

V. Tips on how to manually designate individual tracks

The KymoAnalyzer is very sensitive to user input with regards to the assignment (“clicking”) of tracks. For example, a large number of clicks would generate a multitude of short segments that will affect the calculation of segmental run lengths, segmental velocities and pause-related parameters (***Figure D***).

Thus, for a more accurate assignment of tracks, we recommend to keep the number of clicks to a minimum because, in most of the cases, the resolution is too low to discern whether an apparent slight change in velocity is real or only due to an image acquisition artifact. It is certainly better to reduce the complexity of a track trajectory rather than artifactually add information by adding too many clicks to a track.

Likewise, to compare experimental conditions to each other, we recommend that the manual assignment of tracks is performed by the same investigator.

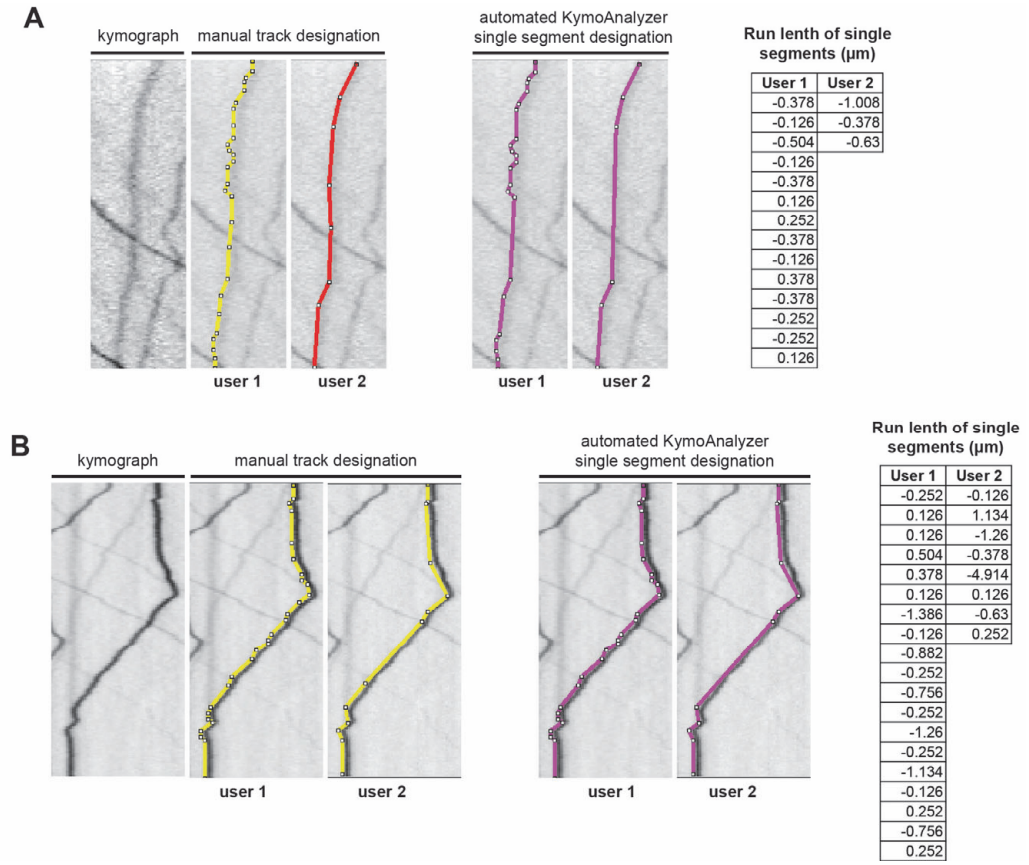


Figure D. Designation of trajectories in kymographs using KymoAnalyzer and variation in the manual designation by two users. (A) and (**B)** show two examples of single track manual designations performed by two users, and the corresponding automated designation of segments within each track by KymoAnalyzer from kymograph images of trajectories of YFP-PrP^{C} vesicle movement. KymoAnalyzer uses the manual track designation as input to calculate segments for each track. White squares within each track represent either user manually-defined “clicks”, or distances between segments as assigned by KymoAnalyzer. To show differences in segment assignment by KymoAnalyzer, two users (user 1 and user 2), independently assigned tracks manually, which led to very different segment assignments and thus to very different calculation of run lengths (tables at the right of (A) and (B)).

VI. Procedure to check for the correct assignment of tracks

The user can check particle trajectory assignment after running the macros. Tracks that may have been missed or misselected can easily be added or modified.

In order to quickly perform a quality control of the particle trajectory assignment, the ***poolData*** macro was designed to generate montage images of the kymographs overlayed with tracks or segments. In the folder “PooledData/Kymographs”, the user can find 3 montage images:

- Kymographs only.
- Kymographs overlayed with track trajectories (colors indicating direction).
- Kymograph overlayed with segment trajectories (note that stationary particles are not traced because they do not contain segments).

Alternatively, the user can check the track assignment on a movie by movie basis, by loading a kymograph (Analysis_Moviename/Kymograph/Moviename.tif), together with the track ROIs (Analysis_Moviename/ROIs/Moviename.zip). The user can adjust any image setting to highlight tracks that may have been missed (brightness and contrast, filtering, thresholding, binarization…). Similarly, particle directionalities, net directionalities and segment assignment can be checked by loading the files CP_ROIs/CP_Moviename.zip, CP_ROIs/NetCP_Moviename.zip and Segment_ROIs/SegmentROI_Moviename.zip, respectively.

If a user needs to add tracks or delete existing tracks on a kymograph, it can be done by running again the Tracks macro on this specific kymograph. Please note that for the changes to be taken into account in the calculation of the parameters, the subsequent macros also have to be run.

VII. Tips on how to modify adjustable settings and thresholds

KymoAnalyzer has been primarily developed to study the transport of fast-moving cargoes (Synaptophysin, Rab3, PrP^C vesicles, etc.), that are imaged at a frame rate of 10 frames per sec (10 Hz), but it is also compatible with cargoes moving with saltatory dynamics (neurofilaments, mitochondria, etc.) that require a lower frame rate of acquisition.

The thresholds d_{max} and v_{pause}, presented below were originally inspired by the ones used in the LAPTrack71, a software that was previously used by our lab and others (2, 3). These values were adjusted following careful visual inspections of several kymographs and hundreds of individual particles of Synaptophysin, Rab3 and PrP^C vesicular transport.
We used the method described above (Part VI) to overlay the clicked tracks and their directionality with the kymograph images (by loading file CP_ROIs/CP_Moviename.zip). d_{max} = 350 nm was the value providing the most accurate assignment of mobile versus stationary tracks, by comparing with what we would have done if we had to do it manually. To increase the sensitivity of the detection of mobile tracks versus stationary, this value can be decreased. The pause detection threshold v_{pause} was set to 0.1 µm/sec. This value is identical to the one incorporated in the software LAPtrack71. We performed a visual observation of dozens of particles trajectories overlayed with their segments (Segment_ROIs/SegmentROI_Moviename.zip) and checked that the number of pauses detected by the software was compatible with what we would have done if we had to do it manually. This quality control procedure confirmed that v_{pause} = 0.1 µm/sec provided an accurate detection of pauses with our temporal resolution of 10 Hz.

Please note that a multiplication factor is integrated in the KymoAnalyzer to automatically adjust the thresholds depending on the user movie frame rate (frames/sec) and image pixel size (µm).

Depending on the pattern of mobilization of their cargo of interest, users may want to adjust some settings and thresholds of the KymoAnalyzer software. They can also contact our laboratory with inquiries and suggestions about how to optimize KymoAnalyzer to their requirements.
Below are the parameters that can be adjusted by users. To open and modify a macro, drag the .ijm file corresponding to the macro into ImageJ. A console window with the macro editor will open, which the user can modify for the following parameters:

1. **Threshold for the detection of mobile versus stationary tracks (d_{max})**

This threshold is the distance deviation from the track center (set by default to 350 nm; ***Supplementary Figure 2***). If the maximum deviation is greater than 350 nm, the vesicle is considered mobile. To increase the sensitivity of the detection of mobile tracks versus stationary, this value can be decreased. This value may be increased for studying particles moving with low velocity.

In the ***CargoPopulation*** macro go to code line 179:

```
cmin=0.35;
```

Change the purple number 0.35 to the desired threshold. Value is in µm.

Do the same in the ***NetCargoPopulation*** macro (code lines 152 and 165).

2. **Threshold defining a switch (change in direction)**

Set by default to 350 nm.

In the ***CargoPopulation*** macro go to code line 197:

```
cminRV=0.35;
```

Change the purple number 0.35 to the desired threshold. Value is in µm.

3. **Change default frame rate and pixel size**

In the macros ***CargoPopulation***, ***NetCargoPopulation*** and ***Segments*** a window pops up and prompts you to enter the pixel size (µm) and frame rate (number of frames/sec) of acquisition. These fields are prepopulated with the imaging parameters used in our lab.

To modify the default pixel size, open the ***CargoPopulation*** macro and go to code line 21:

```
Dialog.addNumber("Pixel Size in µm",0.16);
```

Change the purple number 0.16 to the pixel size of your movies. Value is in µm.

To modify the default frame rate, open the ***CargoPopulation*** macro and go to code line 22:

```
Dialog.addNumber("Frame Rate in /sec", 10);
```

Change the purple number 10 to the frame rate of your movies. Value is in frames/sec.

Do the same in the ***NetCargoPopulation*** macro (lines 10 and 11) and the ***Segments*** macro (code lines 17 and 18).

4. **Threshold for the assignment of segments/pauses (v_{pause})**

This threshold is the minimal velocity for a segment to be considered motile (set by default to 0.1 µm/sec). If the velocity of a segment is lower than 0.1 µm/sec, it is considered a pause. This threshold has to be modified when studying slower-moving particles or when imaging at a lower frame rate. In order to consider the temporal resolution of the movies (frame rate) in the detection of pauses, we integrated a multiplication factor in the KymoAnalyzer ***Segments*** macro.

```
pause threshold = 0.1/factor
```

This factor allows the automatic adjustment of the pause velocity threshold based on the frame rate. It is set at “10/frame rate”, meaning that with our default frame rate of 10 frames/sec, factor = 1 and pause threshold = 0.1 µm/sec. So, if your movies have a frame rate of 0.5 frames/sec, factor = 20 and pause threshold = 0.05 µm/sec, meaning that the pause detection is 20 times less sensitive.

To modify the pause velocity threshold, open the ***Segments*** macro and go to code line 192:

```
if (abs(SVTemp[i])<0.1/Factor && abs(SVTemp[i+1])<0.1/Factor){
```

Change the purple numbers 0.1 to the desired pause velocity threshold. Value is in µm/sec.

Do the same in code lines 391, 416, 426, 439, 447, 494, 523, 552, 583, 588, 593, 594, 598, 601.

Instead of changing all the threshold values, you can modify the multiplication factor. On the other hand, note that if you modify the pause velocity threshold values and your movies have a frame rate different from 10 frames/sec, you must change the multiplication factor accordingly.

To modify the factor open the ***Segments*** macro and go code line 22:

```
Factor=10/Frame;
```

Change the purple number 10.

References

1. Schindelin J, Arganda-Carreras I, Frise E, Kaynig V, Longair M, Pietzsch T, et al. Fiji: an open-source platform for biological-image analysis. Nature methods. 2012;9(7):676-82. 10.1038/nmeth.2019
2. Reis GF, Yang G, Szpankowski L, Weaver C, Shah SB, Robinson JT, et al. Molecular motor function in axonal transport in vivo probed by genetic and computational analysis in Drosophila. Mol Biol Cell. 2012;23(9):1700-14. 10.1091/mbc.E11-11-0938
3. Encalada SE, Szpankowski L, Xia CH, Goldstein LS. Stable kinesin and dynein assemblies drive the axonal transport of mammalian prion protein vesicles. Cell. 2011;144(4):551-65. 10.1016/j.cell.2011.01.021