**User Manual Scission\_Analysis**

**Data acquisition**

Movies are sequences of fluorescent images from cells transfected with a pHluorin (or SEP) tagged receptor (most often the transferrin receptor, TfR) and possibly with a second, red fluorescent protein. Movies are produced by the Metamorph software as .stk files. Movies are named by the cell number, e.g. 022 or 022-1 or 022-1-5. Hence the filename 022.stk

Every other frame of the movie (odd frames) is at pH 7.4. The even frames are at pH 5.5. The image is either one color or split in two halves, green and red. If there are two colors, there should be an image of beads which are fluorescent in the two colors for image registration (see 3.): beads\_date.tif

**Data processing**

1. Launch **Matlab 2015**. In the command line, type **scission\_analysis**
2. With Matlab, click on ‘1. Cut raw movie’ (cutMovie.m) for 2 color movies, or ‘1.1c’ for single color movies. The Movie is split in frames at pH 7.4 and at pH 5.5 to generate 022\_TfR7.stk (images at pH 7.4) and 022\_TfR5.stk (images at pH 5.5). Likewise, 022\_red7.stk and 022\_red5.stk are generated if applicable.
3. (*optional*). The button ‘1. Play’ permits to play the movie(s) generated. Useful for reviewing the data or for additional analysis later.
4. (*optional*). In the case of 2 color movies, image registration is corrected with an image of beads (obtained the day of the experiment: beads\_date.tif). Click on ‘2. Align bead images’ (chooseBeads2.m). A figure with the two halves of the bead image appears. Clicking on the image will zoom in. Choose one bead on the left side (a nice isolated bead). Click on the ‘Zoom’ button. This will activate the left image. Click on the center of the chosen bead. A red circle will appear. Then click on the corresponding bead on the right side of the image. Repeat this for at least 7 more bead pairs (zooming in and out if needed). Click on ‘Fit and Save’ to align precisely the center of beads to the center of mass of the fluorescent image. Save the ‘coord\_date.txt’ file. Click on ‘Load coordinates’. This will load the fitted bead coordinates. Click on ‘Calculate coeff transform’. Save the coeff\_date.txt for subsequent use in two color movies of the day. To verify the validity of the registration, click on ‘2. Test’ (TestTransform) on the image of beads used previously or on another image acquired the same day. The beads in the two colors should coincide exactly. You can compare the original image by typing testBeadImage in the command line.
5. Launch **Metamorph**. Open 022\_TfR7.stk and 022\_TfR5.stk. Click on ‘Run user program’ and select ‘Multidimensional Image Analysis’.
6. Go back to **Matlab**. Click on ‘3. Clnup’ (cleanup5.m) to… cleanup the list of potential events. A window appears asking for the ‘File with matrix of events’. Select 022\_TfR5\_MIA.trc in …\022\_TfR5.MIA\tracking\. Select 022\_TfR5.stk for ‘Stack of events’. Select …\022\_TfR7.MIA\022\_TfR7.stk for ‘Stack of clusters (TfR7 MIA objects). Adjust parameters for cleanup (defaults should be preferred). Save the file 022\_cln5.trc which contains the list of **candidate events** (here 5 represents the number of frames skipped before the first event is considered, not the pH). Save the 022 candidates(5).xlsx file which contains all the parameters and the candidate and rejected events. Save the 022\_thresholds5.fig file which shows the distribution of parameters used for selection: slope, signal/noise and cluster fraction. Rejected events are in blue, candidate events are in red.
7. Click on ‘4. Browse’ to (browseEventsRnd.m) to review individual events. Select the 2 or 4 movies (TfR5, TfR7, red5, red7), the alignment coefficients (coeff\_date.txt), the matrix of events (022\_cln5.trc). Adjust parameters for event representation (size of the image in pixels, etc…). Click ‘OK’. Two windows appear.
   1. The **top window** is called ‘Browse Events’ and the bottom window ‘Curves event XXX’. The candidate events are presented in a random order. The events have a temporary number assigned, 1 to Nevents (number of candidate events). You can look at the next (previous) event by clicking on the ‘Next’ (‘Previous’) button. You can look at any event by typing its number and clicking on ‘Go’. In the top window three images are displayed. The left one is the average of 5 frames of the TfR7 movies. A true event should have a white cluster in the center. The middle one is the movie generated for this event with TfR5 (usually 20 frames before start and 20 frames after start). The right one is the corresponding image in the red5 movie. The movies will be played simultaneously by clicking on the ‘Frame’ slider or by using the left and right arrow keys of the computer keyboard. At the start of the event (frame 0) a red cross will appear at the center of mass of the detected object (vesicle). This red cross will be visible on frames where the object has been tracked. The red cross will disappear if you click on the ‘Track’ checkbox.
   2. In the **bottom window**, there are successive frames for all four mini-movies, 5 frames before and 10 frames after the start. In the bottom left graph there are the quantifications for TfR7 (dark green), TfR5 (light green), red7 (dark red) and red5 (light red). The solid circles represent quantifications at the images shown, or if tracking is longer, at all the images with a tracked object. If tracking lasts less than 10 frames the circles are hollow when tracking is stopped. Quantification is the average fluorescence in a circle of radius Nc pixels (default 2) centered on the center of mass of the tracked object minus the average fluorescence in an annulus of internal radius Nc and external radius Na (default 5). Before the event quantification is centered at the position of the object at frame 0. After tracking quantification is centered at the position of the last tracked frame.
   3. We now go back on the **top window**. The event can be accepted as a genuine endocytic event or tagged for removal. To do so, click on the ‘Remove’ checkbox and click on the cause of this removal: ‘cluster’ (lack of a preexisting cluster), ‘S/N’ (poor signal/noise), ‘track’ (event is in fact a moving intracellular vesicle or the tracking jumps from a preexisting object), ‘other’ (…). Finally you can write a comment in the ‘comment’ textbox.
   4. **Saving data**. Once you are done with your browsing session, if there are events remaining (ie you have stopped at event XXX), you can click on the ‘removed file: Save’ button. This will save three files: 022\_remXXX.txt, 022\_comXXX.mat, 022\_randXXX.txt which correspond to the files containing the list of events tagged for removal, the list of comments, and the randomization file generated at the beginning. You can also save the list of removed and selected events, the comments and the quantifications on an excel file by clicking ‘WriteXLS’ (it is a good idea to do it before closing the window if the browsing sessions of a given recording are split. The quantifications will be added to the same 022\_browsed.xlsx file. When saving the new xlsx file, click ‘OK’ went asked to replace the file in the ‘Confirm Save As’ question dialog box.)
   5. **Make 022\_clnR.trc file**. Click on ‘RemoveTRC’ button to generate a trc file with selected events.
8. **Generate a cell mask**. Click on ‘1. Play’. Select the 022\_TfR7 movie. Click on ‘MASK:create’ button. This opens a new window. Adjust the threshold by clicking on the slider until you are satisfied with the mask (red pixels). Click on ‘Create Mask’, save the 022\_mask.txt file (and also the mask figure 022\_mask.fig for your record).
9. **Sort events in selected regions**
10. Measure frequency of events
11. Quantify fluorescence in average events