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Supplementary information

Imaging endocytic vesicle formation at high spatial and temporal resolutions with the pulsed-pH protocol

In the format provided by the authors and unedited

User Manual Scission_Analysis

Here is the user manual for analyzing imaging of endocytosis events used in Rosendale et al. Nature Communications (http://www.nature.com/articles/s41467-019-12434-9) and other data produced in the Perrais lab. It describes a step by step procedure using Matlab2018 (using the Image Processing, the Statistics and Machine Learning and the Wavelet toolboxes) and the toolbox scission_analysis available at Matlab Central File Exchange as 72744-scission_analysis.

Another option for segmentation and tracking (step 5.) used in all articles, including Rosendale et al. 2019 is to use Metamorph 32bit with the MIA plugin, developed by Jean-Baptiste Sibarita and Victor Racine. See in Annex for user manual.

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. 092 or 092-1 or 092-1-5. Hence the filename 092-1.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 2018**. 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' (cutMovie1color.m) for single color movies. The Movie is split in frames at pH 7.4 and at pH 5.5 to generate 092-1_TfR7.stk (images at pH 7.4) and 092-1_TfR5.stk (images at pH 5.5). Likewise, 092-1_red7.stk and 092-1_red5.stk are generated if applicable.
3. (*Optional*). The button '1. Play' (play11.m) permits to play the generated movie(s). 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 in two colors (obtained the day of the experiment: beads_date.tif), or two separated images of beads for each of the two colors. Click on '2. Align bead images' (chooseBeads3.m). Define which image (half) is the 'master' (normally the GFP/SEP image), as requested. A figure with the two halves of the bead image (or the two bead images side by side) appears. Clicking on the image will zoom in. Choose one nice isolated bead on the left side. 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). Make sure the choosen beads cover the whole image. 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.m) 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. **Segmentation and tracking of** 092-1_TfR7.stk and 092-1_TfR5.stk. Click on '3. Segment and track' (segMovie.m). It will generate segmented images based on Bspline wavelets and track the objects in successive frames. Use default parameters, and change in priority the threshold for optimal segmentations. Generates 092-1_TfR7seg.stk and 092-1_TfR5seg.stk, movies of the segmented consecutive frames. Note: all the segmented objects are there, including the ones that are not kept for tracking (less than 3 frames long). Generates also 092-1_TfR5.trc (respectively 092-1_TfR7.trc), the list of tracked objects, potential scission events which need to be further validated in steps 6-9.
6. **Selection of candidate scission events**. 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 092-1_TfR5.trc for 'Stack of events'. Select 092-1_TfR7seg.stk for 'Stack of clusters'. Adjust parameters for cleanup (defaults should be preferred). Save the file 092-1_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 092-1 candidates(5).xlsx file which contains all the parameters and the candidate and rejected events. Save the 092-1_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. **Validation of events by a user.** Click on '4. Browse' (browseEvents3.m) to review individual events.
   Alternatively, if you want to randomize the timing of the events which are reviewed (in case of a cell for which changes in event frequency are expected), use '4. Browse Rnd order' (browseEventsRnd.m). See point 9. for specificities.
   Select the 2 or 4 movies (TfR5, TfR7, red5, red7), the alignment coefficients (coeff_date.txt), the matrix of events (092-1_cln5.trc). Adjust parameters for event representation (size of the image in pixels, etc…). Click 'OK'. Two windows appear.
   a. The **top window** is called 'Browse Events' and the bottom window is called 'Curves event XXX'. The candidate events are presented in a random order. The events have a temporary number assigned, 1 to N events (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 and 20 frames after the 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 is tracked. The red cross will disappear if you click on the 'Track' checkbox.
   b. 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 shown images, 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 mass of the tracked object minus the average fluorescence in an annulus of internal radius Nc and external radius Na (default 5). At first, 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.

c. 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.

d. **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 two files: 092-1_remXXX.txt and 092-1_comXXX.mat, which correspond to the files containing the list of events tagged for removal and the list of comments. 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 highly recommended before closing the window when the browsing sessions of a given recording are split. The quantifications will be added to the same 092-1_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.*)

e. **Make 092-1_clnR.trc file**. Click on 'RemoveTRC' button to generate a trc file with selected events.

8. (Optional – instead of 7.) **Browse events in random order** (browseEventsRnd.m). Use as in 7. In Saving data (see 7.d), when clicking of 'removed file:Save' button, you will save one additional file: 092-1_randXXX.txt corresponding to a randomization file generated for this session. The rest is the same as in 8.
9. (*Optional*) Use a Support Vector Machine (SVM) to automatically sort bona fide scission events from false candidate events (*in the various studies we published, we found that about 20% of candidate events are rejected by the user; see 8. and/or 9*.). This tool is developed based on the use of a set of validated data (ie a series of recordings with candidate and validated events with the corresponding 092-1_cln5.trc and 092-1_clnR.trc files) to train a binary SVM classifier. Use '5. SVM Learn' (SVMLearn2.m) to generate a model to sort candidate events with a polynomial kernel (eg Model_Poly_26Cells.mat). We are using a dataset of 9631 candidate events leading to 7763 bona fide events recorded in 26 NIH 3T3 cells. After having generated this model, use '5. SVM Classify' (SVM_Classify2.m) to automatically sort the dataset. It generates a new trc file (092-1_clnSVM.trc).
10. **Generate a cell mask**. Click on '1. Play' (play11.m). Select the 092-1_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 092-1_mask.txt file (and also the mask figure 092-1_mask.fig for your record).
11. **Sort events in selected regions**. Click on '6. Sort Events' (sortEvents2.m). Select the events file and the mask of the cell. It will show all events as red dots in the mask of the cell. Click on 'Create ROI' (alt. Load ROI) to create (load) an ROI to select a subset of events on a region of the cell. Click on 'Sort Events' to sort the events inside or outside of ROI within the cell mask. It creates the files 092-1_clnR_ROI.trc and 092-1_clnR_nROI.trc.
12. **Measure frequency of events**. Click on '7. Freq' (eventFrequencyMo2.m) to measure the cumulative number of events (092-1_freq.fig) and the frequency of events per minute (092-1_histo.fig) stored in 092-1_freq.xlsx. (Optional) Click on '7. Freq/mask' to obtain the frequency per μm^2.
13. **Quantify fluorescence in average events**. Click on '8. Quantify Fluo' (avFluo4.m). It generates 092-1_TfR5.fig, 092-1_TfR7.fig, 092-1_dyn5.fig and 092-1_dyn7.fig, as well as 092-

1_events.xlsx which stores all parameters and measures. You can edit a script with '8. Batch' (edit avFluo_calc) to quantify all four channels at once.

14. **Interleave and correct** Click on '9. Interleave and correct red' (interleave_Correct.m) to obtain to obtain a single measurement curve for the two measures at pH 5 and pH 7 of the red channel, taking into account bleed-through from green to red. If you choose 'auto' as bleed-through coefficient (default) it will determine automatically the coefficient which minimizes the difference (sum of squared differences) between average values at both pH). This minimum is shown in 092-1_bleedthrough40.fig. The interleaved corrected fluo values are shown in 092-1_dyn.fig and stored in the spreadsheet 092-1_dyn_deint in the 092-1_events.xlsx file. We obtain values of 3-4% on our imaging setups.
15. **Classify terminal / non terminal** Click on '10. Classify term / non term' (termVSnonterm.m) to sort events into terminal (TfR7 fluorescence decreases to background values, i.e. CCS disappears) and non-terminal events (CCS does not disappear). It evaluates the tfR7 fluorescence values with the following default parameters: average on 4 frames at scission (frames -3 to 0) and 9-12 frames after scission. The event is considered terminal if the ratio between values is lower than 0.4 and non-terminal if greater than 0.6.
16. **Randomization** Click on '11. Randomize' (randomization(200)). It generates confidence values for fluorescence measurements. It shifts the event coordinates in random directions and distances (within the cell mask) for all measures 200 times and calculates 95% confidence intervals. You must use the same parameters as the ones used for fluorescence measures (step 13.) and the pH of 1st frame, bleed-through coefficient, and time interval used for step 14. It generates 092-1_rand(200).fig, showing all three traces (TfR7, TfR5 and red protein) with the confidence intervals (95% up, median, 95% low), and 092-1_rand(200).xlsx which will be necessary to pool several cells (step 18.).
17. **Create ministacks** Click on '12. Create ministacks'. A ministack is a movie centered on a selected events (for example with Browse events, step 7.). Very useful to show a series of images to illustrate an event in a Figure. You can create ministacks for several events named by their number separated by commas.
18. **Pool cells** First, put the XXX_events.xlsx and XXX_rand(200).xlsx files for each cell in a dedicated folder. Select this folder as the default Matlab folder. Click on '13. Pool Cells' (poolCells.m). It will generate a graph Pooled_red.fig and a data file Pooled_red.xlsx pooling all events from the cells. PoolCells will only work if for every cell the fluorescence quantification, interleave and correct, histogram, classify terminal / non terminal, and randomization (i.e. steps 13-16) have been performed for all cells with the same parameters.

Annex 1: List of m files used

Called in scission_analysis	complementary
cutmovie	stkread
cutmovie1color	stkwrite
play11	imread_dp
chooseBeads3	pixvalm
TestTransform	mzoom
testbeadImage	trackIDL
segMovie	loadData4SVM
cleanup5	loadEvents2
SVMLearn2	loadEvents2L

SVM_Classify2	ev2ev
browseEvents3	numinputdlg
browseEventsRnd	dlmread_dp
sortEvents2	Rainbow lookup table
cln2cln	rainbow
eventFrequencyMo2	checkRegs
normFreqMo	findSD
freqByMask	QuickSeg2
avFluo4	Seg_conn
avFluo5	shifSWC
avFluo_calc	sigmaclip
interleave_Correct	SWC_via_subtraction
termVSnonTerm	watershed_WC
sortEvents2	
randomization	
makeMinistacks	
poolCells	
poolFreq	

Annex 2 – alternative to step 5 with Multidimensional Image Analysis, plugin for Metamorph 32bit. It runs faster and is more user friendly than segMovie.

Launch **Metamorph**. Open 092-1_TfR7.stk and 092-1_TfR5.stk. Click on 'Run user program' and select 'Multidimensional Image Analysis'. Parameters to use in MIA (stored in profile.pfl).
'Seg on Details' Tab: Use plans <2 & 2-4, with >0 Threshold, Value 6, Operator Union. Watershed on the result 5.
'Options\Statistics' Tab: Write statistics (on), Binary, Input or on
'Options\Tracking' Tab: Compute tracking (on), Max speed 6, DZ/DX 2, Min life 3, Decrease% 5, Trajectories as ASCII, MM Stack

In addition, Step 6. should be changed as follows: 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 092-1_TfR5_MIA.trc in …\092-1_TfR5.MIA\tracking\. Select 092-1_TfR5.stk for 'Stack of events'. Select …\092-1_TfR7.MIA\092-1_TfR7.stk for 'Stack of clusters (TfR7 MIA objects). Adjust parameters for cleanup (defaults should be preferred). Save the file 092-1_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 092-1 candidates(5).xlsx file which contains all the parameters and the candidate and rejected events. Save the 092-1_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.

Supplementary Table 1 : list of files in Supplementary Dataset

File #	File name	Generated by	Matlab program	Comments	Included
1	beads 160922.tif	acquisition software		two images projected on two halves of the camera. Green on the left, red on the right	Yes
	092-1.stk	acquisition software		Not included (100 Mb). Split in 4 by cutmovie.m	No
2	092-1_TfR5.stk	Cut raw movie	cutmovie.m		Yes
3	092-1_TfR7.stk	Cut raw movie	cutmovie.m		Yes
4	092-1_dyn5.stk	Cut raw movie	cutmovie.m		Yes
5	092-1_dyn7.stk	Cut raw movie	cutmovie.m		Yes
6	coeffs 160922.txt	Align bead image	chooseBeads3.m	generates the coefficients to transform event coordinates for the red image	Yes
7	092-1_mask.txt	Play/create mask	play11.m	Creates a mask of the cell with simple thresholding. Use TfR7 movie	Yes
8	092-1_TfR5seg.stk	Segment & track	segMovie.m		Yes
9	092-1_TfR5.trc	Segment & track	segMovie.m		Yes
10	092-1_TfR5_segP.txt	Segment & track	segMovie.m	Parameters used for segmentation	Yes
11	092-1_TfR7seg.stk	Segment & track	segMovie.m		Yes
12	092-1_TfR7.trc	Segment & track	segMovie.m	Not treated here but can be used to get information about tracked CCSs	Yes
13	092-1_TfR7_segP.txt	Segment & track	segMovie.m	Parameters used for segmentation	Yes
14	092-1_cln5.trc	Cleanup	cleanup5.m	Uses the criteria for a bona fide event (S/N, slope, preexisting clutse) to keep vesicles tracked in 092-1_TfR5.trc. 5 in 'clnR' refers to the number of frames skipped at the beginning of the movie.	Yes
15	092-1_events5.xlsx	Cleanup	cleanup5.m	Writes spreadsheet 092-1_summary in xlsx file	Yes
16	092-1_thresholds.fig	Cleanup	cleanup5.m		Yes
	092-1_clnR5.trc	Browse	BrowseEvents3.m	Use Browse to visualize and validate or discard events. Not shown in the demo.	No
17	092-1_clnSVM5.trc	SVM Classify	SVM_Classify2.m	Uses a database for validated events based on 26 recordings (not included in the demo)	Yes
18	092-1_freq.fig	Freq	eventFrequencyMo2.m		Yes
19	092-1 histo.fig	Freq	eventFrequencyMo2.m	Last bin of histogram may be smaller than 1 min	Yes
20	092-1_clnSVM20.trc	keep for fluo	cln2cln	A subset of events appearing after frame 20 to quantify fluorescence 20 frames before and after scission	Yes
21	092-1_TfR5.fig	Quantify fluo	avFluo4.m	Writes spreadsheet 092-1 TfR5 in xlsx file	Yes
22	092-1_TfR7.fig	Quantify fluo	avFluo4.m	Writes spreadsheet 092-1 TfR7 in xlsx file	Yes
23	092-1_dyn5.fig	Quantify fluo	avFluo4.m	Writes spreadsheet 092-1 dyn5 in xlsx file	Yes
24	092-1_dyn7.fig	Quantify fluo	avFluo4.m	Writes spreadsheet 092-1 dyn7 in xlsx file	Yes
25	092-1_dyn.fig	Interleave and correct red	interleave_Correct.m	Graph of red fluorescence corrected for bleedthrough from the green channel (4% for this cell) Writes spreadsheet 092-1_dyn_deint in xlsx file	Yes
26	092-1_bleedthrough40.fig	Interleave and …	interleave_Correct.m	Graph of best fit for bleed through coefficient	Yes
27	092-1_TfR5 tnt.fig	Classify term/non term	termVSnonTerm.m		Yes
28	092-1_TfR7 tnt.fig	Classify term/non term	termVSnonTerm.m		Yes

Supplementary Table 1 : list of files in Supplementary Dataset

29	092-1_dyn tnt.fig	Classify term/non term	termVSnonTerm.m		Yes
30	092-1_peaks.fig	Classify term/non term	termVSnonTerm.m	Writes spreadsheet 092-1 histo in xlsx file	Yes
24	092-1_events20.xlsx	Classify term/non term	termVSnonTerm.m	Writes spreadsheet 092-1 averages in xlsx file	Yes
31	092-1_rand(200).xlsx	Randomize	randomization(200)	Creates average fluorescence traces for 200 shifted coordinates	Yes
32	092-1_rand(200).fig	Randomize	randomization(200)	Graph of TfR7, TfR7, dyn along with 95 confidence intervals to show the degree of enrichment of the proteins	Yes