Supplemental Materials

Molecular Biology of the Cell

Saha et al.
Supplemental Information for:

Automated analysis of filopodial length and spatially resolved protein concentration via adaptive shape tracking

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**Supplemental Figure S1:** Flowchart and graphical interface of the image analysis software. *(A)* Flowchart summarizing all features of the image analysis software. Individual operations along the process are divided into mandatory (blue) and optional (green) steps. *(B)* Snapshot of the graphical user interface of the image analysis software. The windows for pre-processing (left panel) and image analysis (right panel) are shown with the position of all buttons for obligatory (blue) and optional (green) steps.
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Automated Tracking Software for Filopodia-Like Structures

User Manual
Version 1.0
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Written and developed in the Galic Lab
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1 Introduction

Filopodia formation, elongation and subsequent retraction are the result of forces emerging from actin polymerization dynamics and membrane tension. The spatio-temporal control of these forces is achieved to a large extent by local and transient enrichment or activation of actin-regulatory proteins during a particular phase of the extension-retraction cycle. Consistently, changes in activity and concentration of individual actin-regulatory proteins that control these regulatory modules have been shown to selectively alter initiation frequency, final length and lifetime of the filopodia, or its retraction. The conclusion from these experiments suggests that the life cycle of filopodia is composed of separate actin-polymerization modules with unique features that transit from one to the next in a stereotypic fashion. However, while many of the proteins that control specific aspects of filopodial dynamics have been described, our understanding of the process has remained incomplete as image analysis software suitable to accurately measure growth dynamics jointly with protein localization in such protrusions has remained elusive. We thus developed an image analysis software that provides automated measurements of filopodia growth dynamics and relative protein concentrations along the filopodial length. A validation of the script using a series of biological samples can be found in Saha T., et al/).

The image analysis software combines analysis of filopodia growth dynamics and information on spatio-temporal protein concentration within filopodia. This is necessary, as proteins that control the filopodia growth show not only a temporal but also a spatial localization along the filopodial length. Importantly, and unlike other approaches such as a line scan, analysis reliably works in flexible filopodia independent of tilting, bending, buckling or lateral shift.

In this manual, we describe the User how to best to apply this tracking software, highlighting relevant parameters that can be modified, but also to discuss limitations of the software. We hope you find the script suitable for your work!
2 WORKING PRINCIPLE OF THE IMAGE ANALYSIS SOFTWARE

Below, you find a short overview of the working principle of the image analysis software that is based on the Convex Hull algorithm present in MATLAB.

2.1 DETECTING THE FILOPODIAL TIP DYNAMICS.

- **Step 1 - outline images:** In case of dual-color images, individual channels are merged to maximize accuracy (Figure 1, top panels). The superimposed image is then binarized to decreases computation load and increase speed and the outline of the structure is determined (Figure 1, bottom panels).

![Figure 1](image)

*Figure 1: Showing the procedure behind making the superimposed mask and the binary outline image.* Here two protein channels (green and red) are shown. First the channels are converted to gray intensity profile. Then using edge detection algorithm, the binary outline image (Intensity value in a pixel is assigned maximum when there is an edge and minimum otherwise) of the superimposed mask is made. The process is repeated for every frames.

- **Step 2 – detect filopodial tip:** A convex hull is generated to encompass the outline generated in Step 1 using the ‘Quick Hull Algorithm’, which is a type of Convex Hull Algorithm. The hypothesis is that one of the protruding structures (i.e. the filopodial tip) will be a vertex belonging to the convex hull (Figure 2a).
Step 3 – track tip throughout the movie: The vertex that belongs to the filopodia tip is then being tracked frame by frame. This is achieved by finding in the subsequent frame the vertex that is nearest to the vertex of the filopodial tip. To avoid wrong annotations due to loss of filopodia tracking, the new vertex in the subsequent frame can only be located inside a specified radius from the initial position (Figure 2b, dotted line). Should no points be detected within the radius, the program considers the filopodial tip as lost, and stops tracking.

Figure 2: Principle behind the tracking algorithm. (a) Convex Hull generated for the outline superimposed mask (b) Tracking of the tip in the subsequent frame (c) Fitting the trace along the filopodia (d) Spatial Protein Concentration Acquisition

2.2 Tracking the body of the filopodia.

Step 1 – connecting tip-to-base: Once the tip of the filopodia is detected for each frame, an imaginary line is drawn between the assumed base point and the tip indicated by the green dotted line in Figures 2b,c.

Step 2 – introducing nodal points: Using this tip-base line, we next introduce nodal points where the line can bend (Figure 2, red dots on the green line). Any number of points can be introduced.
o **Step 3 – scanning fluorescence intensities along nodal points:** At each nodal point the intensity values of the outlined image of the superimposed mask \( I(x_i, y_i) \) (in binary) is measured in perpendicular direction to the line (**Figure 2c**, red lines). Here, \( x_i \) and \( y_i \) represents the \( i^{th} \) pixel’s abscissa and \( i^{th} \) pixel’s ordinate of a perpendicular line.

o **Step 4 – readjusting nodal points:** Follow the position of maximal intensity, the position \([p\bar{x}, p\bar{y}]\) of the nodal points are then updated according to the formula shown below.

\[
[p\bar{x}, p\bar{y}] = \left[ \frac{\sum_i I(x_i, y_i) \times x_i}{\sum_i I(x_i, y_i)}, \frac{\sum_i I(x_i, y_i) \times y_i}{\sum_i I(x_i, y_i)} \right]
\]

o **Step 5 - creating filopodial skeleton:** The newly positioned nodal points are then joined to approximate the shape of the filopodia in each frame represented by the ‘red line’ joining the ‘red dots’ in **Figure 2c**.

### 2.3 Spatial Protein Concentration along the Approximated Shape of the Filopodia

o **Step 1 – get filopodial fluorescence intensity:** At each pixel along the trace, an imaginary line perpendicular to the local direction of the trace is drawn (**Figure 2d**). The highest 3 intensity values are then averaged to estimate the protein concentration at each height position along the trace of the filopodia.

### 2.4 Ratiometric Analysis

o **Step 1 – get normalized ratiometric data:** For spatially comparing two fluorescence proteins in a symmetric manner increase and reduction are both, the ratio of the corresponding protein intensities is visualized using a log scale for every pixels along the trace. This is achieved in two steps:

  - In a re-normalization step, intensity values are first rescaled to values from 0 to 9 and then shifted such that the protein values range from 1 to 10.
  - \( \log_{10}\left(\frac{\text{Protein of Interest}}{\text{reference protein}}\right) \) is then applied to the ratio of the re-normalised protein channels for quantitative visualization of the relative protein concentration along the trace.

o **Why log scale?** Let’s assume we have two proteins, called A and B. In one case, A is enriched 8-fold over B and in the other case B is enriched 8-fold over A. Showing the
ratio as a linear scale of A/B will yield data where these two cases do not have the same distance from the equilibrium (concentration A=B). In contrast, using log(A/B) will make the distance equal.
3 INSTALLATION AND SPECIFICATIONS

3.1 HARDWARE
Considering that during the analysis temporary data ~4 times the size of the single channel image file is stored in memory (RAM or virtual RAM), sufficient RAM should be allocated. Having sufficient RAM will also increase computational speed as it reduces load on virtual RAM, which is comparatively slow.

3.2 SOFTWARE
The program has been developed and used in MATLAB R2015a and R2015b, which we recommend to use. Previous versions (2010b or above) can be used, but the full functionality of the script is not ensured. For editing or pre-processing the stacked ‘.tiff’ files, which is optional, we recommend using ImageJ or Fiji.

3.3 INSTALLATION
Download the software package from github (note: the link will be made available upon acceptance) and unzip all the files into your work folder. All the input and output files must be in this work folder. Please do not to change in the work folder the name of the Excel file that is included or the name of individual sheets in the Excel file. Changing the name of the Excel sheets and the file will cause an ERROR message.
4 CONSIDERATIONS CONCERNING IMAGE QUALITY

A number of parameters should be considered when acquiring images:

- First, filopodia should not leave the acquisition window. To avoid analysis of such out-of-focus artifacts, which would result in large jumps in filopodial length, we introduced a parameter defining the maximal allowed tip displacement between frames (Figure 2, dotted circle). Note that the size of the radius can be changed (Figure 3 button <6d>). How this is done will be described below in the ‘Using the GUI’ chapter.

- Second, as the base of an elongating filopodia is defined only in the initial image, drift should be corrected prior to image analysis (e.g. https://github.com/NMSchneider/fixTranslation-Macro-for-ImageJ).

- Third, to minimize noise-based errors, we recommend a signal to noise ratio of more than 4 for filopodial structures.

- Fourth, we recommend to keep pre-processing (e.g. changing brightness and contrast) of the uploaded image series to a minimum as this may introduce errors (e.g. saturation of pixel values that will introduce errors in the ratiometric image analysis).

- Fifth, as the image analysis software does not correct for bleed-through between individual channels, the compatibility of fluorescence probes and filters should be tested prior to image acquisition.

- Sixth, as these thin structures are depending on cell type 2-4 μm in length (Portera-Cailliau et al., 2003; Mogilner and Rubinstein, 2005), we recommend using at least a 60x objective to accurately assess growth dynamics and relative fluorescence intensity.
5 USING THE GUI

5.1 OVERVIEW

(a) Logical flow and general steps involved in using the GUI. The blue steps are the primary steps that are mandatory, and the green steps indicate some secondary optional features for better analysis. (b) Showing all the buttons present in the two GUI windows that are involved in steps described above. GUI window #1 is called ‘Load Channel’, GUI window #2 is named ‘Analysis Window’.

Figure 3: GUI of image analysis software. (a) Logical flow and general steps involved in using the GUI. The blue steps are the primary steps that are mandatory, and the green steps indicate some secondary optional features for better analysis. (b) Showing all the buttons present in the two GUI windows that are involved in steps described above. GUI window #1 is called ‘Load Channel’, GUI window #2 is named ‘Analysis Window’.
• **Step 1 – loading the movies:** Movie files for individual channels are uploaded separately (Figure 3, button <1a> to <1d>). Uploaded media files should be in stacked ‘.tiff’ file format. After loading, the User can select several optional features (Figure 3, green buttons). For instance, the region of interest can be cropped, enhanced and rotated (Figure 3 button from <2e> to <2i>). Undesired regions, such as sections from other cellular structures that could affect the convex hull analysis, can be deleted (Figure 3 button <2j>). Contrast and brightness of individual channels can be separately adjusted (Figure 3, <2d> to <2f>). The final, processed images are stored in stacked ‘.tiff’ 8-bit gray image file format in the work-folder. For consistency, we recommend to check all frames (Figure 3. Button 2c) and confirm that the images are not saturated or drifting. If needed, all image corrections can be deleted using the ‘reset’ button (Figure 3, button <1e>).

• **Step 2 – generate outline:** Once the channels are loaded and the image is processed as desired, click on ‘TrackingWindow’ button (Figure 3, button <3a>) to open the window #2 for image analysis. After the analysis window #2 opens, click on ‘Boundary’ button (Figure 3, button <4>) to generate the outline of the superimposed mask of the cell body. Next, the outline profile of the superimposed mask can be played (Figure 3, using the slider in window #2) to determine the position for the initial reference points (Figure 3, button <5>). To optimize the performance of the image analysis script, all tracking parameters can be modified as desired (Figure 3, button group 6 (for detailed description of parameters, please check the section ‘Introducing the Buttons Step by Step’)).

• **Step 3 – tracking the filopodia:** Now, the program is equipped to track the filopodium. After initiation of filopodia tracking (Figure 3, button <7>), the process of tracing is shown in real-time during the runtime of the program. Approximated filopodial length (red) are stored in a separate Excel file in the sheet named ‘length_vel’. After tracking once, the whole tracking procedure can be viewed by moving the slider clicking on the ‘History trace’ checkbox and ensure that the trace is almost always above the filopodia for better analysis.

• **Step 4 – measuring relative fluorescence intensity:** Next, the channels of interest for which intensity values are acquired can be selected (Figure 3, button group 8). It is possible to either determine the intensity along the full length of the filopodium (Figure 3, button <8b>) or only
for specific number of pixels at the leading tip (Figure 3, button <8f>). If the latter is chosen, relative size and position within the filopodium need to be determined (Figure 3, buttons <8g>, <8h>). This is then repeated for each channel, and the protein intensity values are saved in memory and the Excel file before the comparison by ratio calculation is initiated (Figure 3, button group 9).

- **Step 5 – generating the ratiometric image:** Finally, the relative concentration of two individual channels can be selected (Figure 3, button <9b> and <9c>) and plotted. All protein intensity values are stored for further validation in the Excel file. A demonstration of the image analysis software can be found in Movie 9. For more details on the image analysis algorithm, please refer to the chapter ‘Working Principle of the Image Analysis’.
5.2 INTRODUCING THE BUTTONS – STEP BY STEP

Figure 4: GUI window #1. Window #1 for loading the Channels corresponding to different Proteins and processing them like changing the brightness contrast levels, removing undesired regions making the movies more compatible for tracking.

5.2.1 Loading the Channels

- Buttons represented by <1a>, <1b>, <1c>, <1d> (see Figure 4) are used for loading the channels corresponding to different proteins. (All channels must have the same dimension (Length × Height × Stacks) with `.tiff` file format)
  - Check in the box corresponding to the push buttons to load the channels into the memory. Up to 3 protein channels can be selected.
Click on ‘CellBody’ button (<1a>) to create the superimposed mask of the Region of interest (ROI) under consideration (The superimposed image is created by selecting the maximum pixel values among the respective pixels of the Protein channels by max(Protein1, Protein2, Protein3) logic).

The superimposed mask can also be made using a different logic. Then Check in the box corresponding to the ‘Cell Body’ to upload the superimposed mask just like a protein channel mentioned before.

**Note:** The User has to select at least one Protein Channel (then it has to be ‘Protein1’) for ‘AnalyseProtein’ (will be discussed later) to work and at least two for ‘Compare’ to work in ‘Window #2’.

### 5.2.2 Removing undesired regions and Making Tracking Compatible

- Buttons represented by <2a> to <2c> and <2d> to <2j> (see Figure 4) are used for making the Superimposed mask more compatible for tracking (alternatively this can be before loading using for instance ImageJ or Fiji)
  - Once all channels are uploaded, buttons <2a>, <2b>, <2c> can be used to choose the frames: the ‘start’ frame is selected using the middle slider <2a> and the ‘end’ frame is selected by the bottom slider <2b>. Button <2c> can be used to view each frames of the movie that will be used for analysis in Window #2.
  - Button <2h> can be used to crop out the region of interest (using a ‘rectangle’ tool). Nearby outgrowing structures must be avoided for smooth tracking.
  - Button <2i> can be used to ‘rotate’ the ROI (for consistency, we keep in our experiments the filopodia tip vertically up). Unwanted regions can also be deleted using the ‘delete’ button. Clicking on the ‘delete’ button will open a pointer to draw a polygon around the region to be deleted in all of the Superimposed Mask (Protein channels are not affected). (**Note:** please ensure that the filopodia will at no point enter the region that will be deleted, as the signal in the selected region is not only deleted in a single frame but in all frames of the movie.)
  - Buttons from <2d> to <2f> can be used to adjust the contrast (or controlling the Pixel Value range in each channels) of the superimposed mask and the Protein channels.
- Button <1e> can also be used to reset the changes made to the images to the ‘raw’ image files uploaded initially using <1a> to <1d> buttons.

**Note:** Please feel free using third party software like ImageJ, Fiji to preprocess (e.g. brightness contrast level, cropping) the images before uploading.

### 5.2.3 Generate the trace

- Button <3a> is used for transition into the Window #2 (Analysis Window) for Analysis
  - Once everything is optimized, the User can click on the ‘TrackingWindow’ button <3a> for opening the Window #2.
  - Using <3b> the User can enter the ‘Pixel to Micron’ conversion factor to get all the length measurements in ‘microns’ prior to opening the analysis window

![Figure 5: GUI window #2. Window #2 for tracking and analysis. In this window all tracking parameters are assigned. Tracking is executed and the spatio temporal trace (the kymograph) is stored in memory which is further used for relative spatial protein concentrations.](image-url)
Clicking on the ‘TrackingWindow’ button (<3a>) in Window #1 opens Window #2 (Shown above in Figure 5)

- In Window #2 the User should click on the ‘Boundary’ button <4> to generate the outline of the superimposed mask used for tracking.

- In order to successfully analyze the filopodium, it is necessary to check for a convex hull point near the tip in each frame using the slider (since a vertex in the convex hull is used as an identifier of a tip by the program). Once the tip is defined, the User can move to the next step. If this is not possible, the User has to re-crop or delete undesired regions going back to Window #1. **Note: if the tracked filopodia is crossing other objects, gets out of focus during the movie, or has a low signal-to-noise tracking accuracy is impaired.**

- Then two reference points on the ‘Image Window 1’ has to be selected by a single click on ‘Reference’ button <5>. First, the origin from where measurement is made is determined and then the tip. By doing this, the User defines what Convex hull point will be the future filopodial tip point.

- The parameters for tracking have to be assigned using the blank box represented by <6a>, <6b>, <6c>, <6d>, <6e>:
  - ‘No of segments’ <6a> determines the number of nodal points where the kymograph (or the trace line) can bend to fit the filopodia structure.
  - ‘Scan Width’ <6b> determines the width of the region at each nodal points that is scanned to track or catch the bending filopodia body.
  - ‘Accurate Meas. after’ <6c> determines the length along the trace above which the kymograph can bend.
  - ‘Radius of tip detection’ <6d> represents the permitted radius, or ‘tip detection circle’ inside which the tip (= nearest convex hull point) of the filopodia will be detected in the next frame.
  - ‘Angle Threshold’ <6e> represents the extreme angle of the cone where the filopodia can wiggle and bend. If the filopodia tip goes beyond that range then the program holds the tip position where it has left before until it finds any convex hull point inside the ‘tip detection circle’.
For better tracking some additional features are introduced for manual adjustments represented by <6f>, <6g>, <6h>, <6i>, <6j>, <6k>. These features help the tracking algorithm to trace the filopodia in case of lost tracks (e.g. due to bad SNR ratio). The User can use these buttons to assist the algorithm by setting some extra tip reference point and parameters in some specific frames for continuous tracking and data acquisition where the automated algorithm fails to find the tip.

- Once the tracking program <7> is executed, the User can look into the blue regions ‘no tracking region’ in ‘Image window 2’. Using the slider, the frame where the automated algorithm lost track can be viewed and the User can assign a new reference tip point (a convex hull point if there is any on the actual tip of the filopodia) using the button ‘SelectReference’ button <6f>. If not, the User can manually hold the position of the tip of the kymograph accordingly. It is also recommended to ‘check in’ the box in the button group represented by <6l> to look at the trace with overlay of the ‘superimposed mask’ in ‘Image Window 1’ in that frame.

- Ich changes were made, the User should also specify other parameters such as ‘Accurate Meas. after’ <6g> and ‘Radius of tip detection’ <6h>. (Note: Keeping these parameters blank will give an Error) in that frame and click on the ‘Add’ button <6i> to register the parameters of that frame in the memory.

- The User can also delete the parameters stored in that frame using the ‘Delete’ button or delete the manual parameters set in all frames using ‘Reset’ button in the button group represented by <6k>.

- The User should then again click on the ‘Track&Analyze’ button <7> to generate the new kymograph (or trace) using the newly added information and the data is automatically saved in disk (.xlxs) for further reference.

- If the User wants to repeat the tracking, simply reload the stored parameters from the memory using the ‘ImportParameters’ button in the button group <6l>.

### 5.2.4 Protein Tracking and Ratiometric Analysis

- Once the trace has been generated, the User can click on ‘Analyse Protein Intensity’ represented by <8a>.
The User can select the boxes represented by <8b>, <8c>, <8d>, <8e>, <8f>, <8g>, <8h>, <8i> depending on the requirements:

- If the User wants to detect the spatial distribution of proteins along the full filopodia, select ‘Whole filopodia’ <8b> and the Proteins that will be tracked using <8c>, <8d>, <8e>.

- If the User wants to detect the protein intensities only at the tip select the box ‘Leading tip’ <8f> and the size of the tip (in Pixels) using the box ‘Tip length’ <8g>. In that case, the User also needs to specify the length (in Pixels) in the box named ‘threshold’ <8h> above which the tip measurement should start. **Note:** The tip length must be always less than or equal to threshold.

Once the protein intensities are registered in the memory, the User can use the ‘Compare’ button <9a>. The User needs to specify the ratio (in log) <9b> and or <9c>. **Note:** The User should at least analyse two proteins for the compare function to work as mentioned before.
5.3 A GUIDE STEP BY STEP (WITH SAMPLE VIDEO)

- **Step 1 - Initiate**
  - Open Matlab (version R2015a/b recommended) by typing Matlab as shown below
  - Browse into the work folder. Then in the Command Window type ‘filoAnalysisM3’ and press <ENTER>
- This will open the GUI for Window #1

- **Step 2 - Load and modify**
  - Now say we have two protein channels, GFP-cytosol marker as reference (i.e. Protein1) and RFP-actin marker (i.e. Protein2). The boxes corresponding to Protein1 and Protein2 are checked, and the videos (stacked tiff files) are loaded accordingly by clicking on the corresponding protein channel buttons (after clicking on the buttons, please wait until the images pop up on the screen as shown below).
- Click on ‘CellBody’ to create the superimposed stacked mask from the two protein channels and wait until the cell mask pop up as shown below.

![Image of cell mask creation process]

- The User can now rotate and make the filopodia upright (recommended but not necessary) using the rotate button as shown below. Here the User has to select the tip of the filopodia (marked by ‘yellow line’ on the superimposed mask) followed by assigning the final direction (marked by the ‘blue line’) and wait until all the channels get rotated as shown below.

![Image of filopodia rotation process]
The User can now directly click on ‘TrackingWindow’ to move to the analysis window for generating the trace.

**Step 3 - Generating trace and Ratiometric analysis**

Upon clicking on ‘TrackingWindow’ wait until the GUI Window #2 pops up (as shown below). *(Note: may take some time. If Window does not appear, please check for any errors in the command window).*
- The outline of the superimposed mask is generated by clicking on ‘**Boundary**’ as shown below.

- Check for undesired convex hull points (if any are present, please use GUI Window #1 to delete those regions). Also, ensure to have a convex hull point in every frame at the tip of the filopodia with the help of the slider.

- Then select the reference point as shown below. First the origin (green circle) and then the tip (red circle) as described earlier.
Also the general parameters are assigned prior to the tracking. Tracking is then initiated using the button ‘Track&Analyze’. Wait until the tracking is over since the tracking history is stored after it is completed for further assistance and reference.

For spatial protein information along the trace in each frame the box corresponding to each protein is checked in as shown below and ‘Analyze Protein Intensities’ is clicked.
o For leading tip analysis the ‘Tip Length’ and ‘threshold’ is registered into the memory as shown below. The box corresponding to the ‘Leading tip’ is also checked.

o For spatially comparing the User has to check the box (in our case Protein B by Protein A) as shown below (as there is only protein B and A) and click the ‘compare’ button.
Also for comparing the intensity in the leading tip the User has to check the boxes as shown below.

- **Step 4 - Smooth tracking (additional features) shown using a different movie**
  - The User can use the slider and the cursor (green cursor moves with slider in ‘GUI Window 2’) to check for points where the program lost track (generally the program does not track when there is no convex hull point inside the detection circle (at the filopodial tip) which is indicated by blue region in ‘GUI Window #2’) In this case, the User can reassign the tip and regenerate the trace.
5.4  TROUBLESHOOTING

5.4.1  Errors encountered during Inputs

Errors encountered during loading images:

- Check: If the images are in gray scale stacked tiff file format (Not in RGB or other formats).
- Check: All the protein channels must be of same dimension (Length × Height × Stacks).
- Ensure that all input files are in the work folder (Note: The work folder must be the current folder in MATLAB).

5.4.2  Errors encountered during output

Error encountered while writing data to file:

- Check: Enough disk space.
- Check if the Excel sheet was open while the program was running. It is mandatory to close the Excel and all input files (including stacked image files) while the program is running.
- Ensure not to change the name of the excel and other input files.
6 IMAGE FILES FOR TESTING THE SOFTWARE

6.1 GETTING TO KNOW THE SOFTWARE (IN SILICO TESTS)
Here we provide some movies for testing the software using the GIU

- **Movie 1.** Simulation of a filopodium emerging from the dendritic shaft. The filopodium is emerging and retracting perpendicular to the dendrite

- **Movie 2.** Simulation of dendritic filopodia with different signal/noise ratios. Filopodia with varying gray values (from left to right, average = 100, 80, 60 and 40; variance = 10) and a constant background noise (average = 10; variance = 10) are shown

- **Movie 3.** Simulation of tilting filopodium. An extended filopodium is tilting to the side and back.
• **Movie 4.** Simulation of filopodium growing at an angle of 45 degrees. Filopodium is emerging and retracting from the dendrite at a constant angle of 45 degrees.

![Movie 4](image)

• **Movie 5.** Simulation of curved dendritic filopodium. Curved filopodium is emerging and retracting from the dendrite.

![Movie 5](image)

• **Movie 6.** Simulation of filopodium with protein enrichment along the whole filopodial length. Dual-color movie depicting enrichment of protein (red) over reference (green) in filopodium during the whole extension-retraction cycle.

![Movie 6](image)

• **Movie 7.** Simulation of filopodium with protein enrichment in the extending tip. Dual-color movie depicting enrichment of protein (red) over reference (green) in the filopodial tip during extension.

![Movie 7](image)
• **Movie 8.** Simulation of filopodium with protein enrichment in the retracting tip. Dual-color movie depicting enrichment of protein (red) over reference (green) in the filopodial tip during retraction.

![Movie 8](image)

• **Movie 9.** Example showing about how to use the GUI with a dual color filopodium stacked tiff file of a HeLa cell.

![Movie 9](image)
7 Acknowledgements

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8 Correspondence

Please feel free to ask any questions (contact information below). Any suggestions for further developing the software are welcome.

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