Video Article

A Graphical User Interface for Software-assisted Tracking of Protein Concentration in Dynamic Cellular Protrusions

Tanumoy Saha[1], Isabel Rathmann[1], Milos Galic[1]

[1]DFG Cluster of Excellence 'Cells in Motion', (EXC 1003), Institute of Medical Physics and Biophysics, University of Muenster

Correspondence to: Milos Galic at galic@uni-muenster.de

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Abstract

Filopodia are dynamic, finger-like cellular protrusions associated with migration and cell-cell communication. In order to better understand the complex signaling mechanisms underlying filopodial initiation, elongation and subsequent stabilization or retraction, it is crucial to determine the spatio-temporal protein activity in these dynamic structures. To analyze protein function in filopodia, we recently developed a semi-automated tracking algorithm that adapts to filopodial shape-changes, thus allowing parallel analysis of protrusion dynamics and relative protein concentration along the whole filopodial length. Here, we present a detailed step-by-step protocol for optimized cell handling, image acquisition and software analysis. We further provide instructions for the use of optional features during image analysis and data representation, as well as troubleshooting guidelines for all critical steps along the way. Finally, we also include a comparison of the described image analysis software with other programs available for filopodia quantification. Together, the presented protocol provides a framework for accurate analysis of protein dynamics in filopodial protrusions using image analysis software.

Video Link

The video component of this article can be found at https://www.jove.com/video/55653/

Introduction

Spatio-temporal control of actin regulatory proteins is associated with filopodium dynamics [1,2]. Tracking spatially resolved protein concentration along the whole filopodial length through time is thus crucial to advance our understanding of the mechanisms underlying initiation, elongation, stabilization or collapse of these dynamic structures [3,4]. Unlike protein analysis in the cytosol, where many cell shape changes occur at a larger scale, filopodia are dynamic micro structures that constantly buckle [5] and bend, thus precluding analysis using a simple approach such as a line-scan.

Different software solutions for tracking filopodial shape are available [6,7,8,9]. Likewise, software for ratiometric tracking of protein dynamics within the cell body has been developed [10,11]. To combine automated tracking of filopodial shape and spatio-temporal protein analysis, we recently developed an image analysis software based on the convex-hull algorithm [12]. This novel analysis method, which is operated *via* a graphical user interface (GUI), combines for the first time, relative protein concentration along the filopodial length and growth velocity, thus allowing the accurate measurement of spatio-temporal protein distribution independent of movement of these dynamic structures [12].

The idea behind the software (source code is freely available, see below) is that one of the vertices of the convex hull will coincide with the tip of the filopodium (**Figure 1A**). By looking in the subsequent frame for the nearest vertex of the convex-hull, the moving tip can be tracked throughout the whole movie. Once the tip is detected in each frame, its position is used to draw an axis by joining the tip with a reference point at the base of the filopodium (**Figure 1B**). Finally, using equidistant nodal points, whose positions are determined by the median pixel with maximum intensity along the line orthogonal to the axis, are used to determine a backbone that follows the filopodial shape. Taking advantage of this adaptive backbone, a kymograph is generated to trace filopodial growth and protein concentrations for up to three channels along the filopodial length (**Figure 1C**).

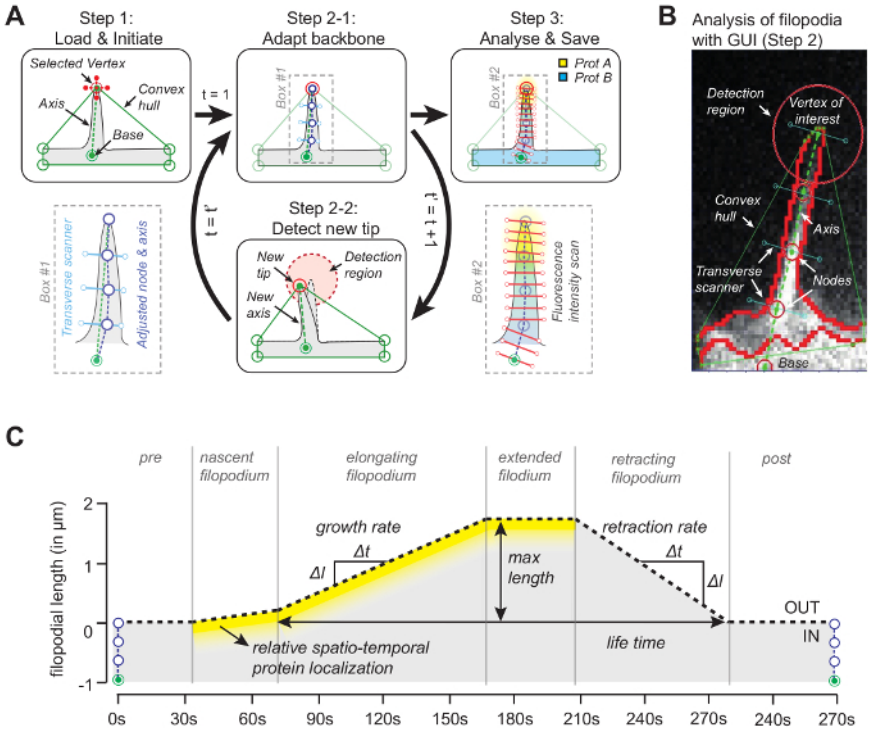

Figure 1: Working Principle of the Image Analysis Software. (**A**) The algorithm behind the software. In Step1 the user specifies the reference (base) and the vertex (the tip) of the filopodium. In Step 2-1 the backbone of the filopodium is obtained using the median pixel with maximum intensity value. In Step 3 the backbone is used for spatial protein intensity profile. In Step 2-2 the software automatically tracks the tip in the subsequent frame. The whole procedure iterates. (**B**) Snapshot of the algorithm with real filopodium introducing important elements such as the convex hull that is being used for tracking. (**C**) Overview of parameters that can be measured with the algorithm. This figure has been modified from reference [12]. Please click here to view a larger version of this figure.

The image analysis software is operated in Matlab (referred to as programming software) via a graphical user interface. To maximize flexibility and robustness for the particular experimental setting, the user can adjust a series of tracking parameters (*e.g.* permitted bending angle and inter-frame movement) and also make some corrections to the movies (*e.g.* cropping, rotation, removal of unwanted objects) (**Figure 2A** and **Table 1**).

GUI	No.	Mandatory	Description	Name (in GUI)
#1	1a	Y	Loading stacked .tiff file representing cell body (with box checked in) or create superimposed cell body from channels	CellBody
#1	1b	Y	Loading stacked image file corresponding to protein 1	Protein 1
#1	1c	Y	Loading stacked image file corresponding to protein 2	Protein 2
#1	1d	Y	Loading stacked image file corresponding to protein 3	Protein 3
#1	1e	N	Resets everything to preloaded stacked image files	Reset
#1	2a	Y	Scroll bar to determine the initial frame for analysis in GUI window #2	NA
#1	2b	Y	Scroll bar to determine the final frame for analysis in GUI window #2	NA
#1	2c	Y	Scroll bar representing current frame	NA
#1	2d	N	The grey value of the pixels below which all pixels will be set to zero	NA
#1	2e	N	The grey value of the pixels above which all pixels will be set to maximum values	NA
#1	2f	N	Set the intensity values of the pixels specified by <2e> & <2f>	Set
#1	2g	N	Play the intensity-adjusted movie	Play
#1	2h	N	Crop Image	Crop
#1	2i	N	Rotate Image	Rotate
#1	2j	N	Delete regions in the whole stack	Delete regions
#1	3a	Y	Click to open the 'Analysis Window' (GUI window #2)	Tracking Window
#1	3b	Y	Enter the size of a pixel in microns	Enter Pixel size
#2	4	Y	Click to generate the boundary/edge image of the superimposed cell body	Boundary
#2	5	Y	Click to select the base and tip of the filopodia	Referernce
#2	6a	Y	Enter the number of segments or nodes	No of segments
#2	6b	Y	Enter the scan length (perpendicular to axis)	Scan Width
#2	6c	Y	Enter the length above which filopodia starts bending	Accurate Meas after
#2	6d	Y	Enter the radius of the tip detection circle (i.e. area where the vertex can be localized in the next frame)	Radius of tip detection
#2	6e	Y	Enter the maximum angle the filopodium can bend from the vertical axis	Angle Threshold
#2	6f	N	Add reference points for base and tip for that specific frame	Select reference
#2	6g	N	Enter the length above which filopodia starts bending for that specific frame	Accurate Meas after
#2	6h	N	Enter the radius of the detection circle for that specific frame	Radius of tip detection
#2	6i	N	After entering all the parameters for the specific frame click to store the values to memory and file for further reference	Add
#2	6j	N	Click to delete the set manually parameters for that frame	Delete
#2	6k	N	Click to delete all parameters stored manually using the 'optional features panel' for all frames	Reset
#2	6l	N	Check in before tracking to store all tracking results in memory for future reference	History trace
#2	7	Y	Click to start tracking	Track&Analyze
#2	8a	N	Click to start tracking protein channel intensity	Analyze Protein Intensities

#2	8b	N	Check in to track protein channel intensity along the filopodial length	Whole filopodia
#2	8c	N	Check in for tracking the reference protein or protein A	ProteinA
#2	8d	N	Check in for tracking the protein B	ProteinB
#2	8e	N	Check in for tracking the protein C	ProteinC
#2	8f	N	Check in to track average protein intensity in the tip	Leading tip
#2	8g	N	Enter the length of the tip	Tip Length
#2	8h	N	Enter the minimum distance from base above which the tip starts forming	threshold
#2	8i	N	Click to save the leading tip analysis results to file	Push Button
#2	9a	N	Click to initiate ratiometric protein analysis	Compare
#2	9b	N	Check in to compare protein B with respect to A	log10(B/A)
#2	9c	N	Check in to compare protein C with respect to A	log10(C/A)
#2	9d	N	Check in to compare protein B with respect to A at the tip	log10(B/A)
#2	9e	N	Check in to compare protein C with respect to A at the tip	log10(C/A)
#2	10a	N	Choose other color-map (default: Jetplot)	Color Map
#2	10b	N	Edit the colormap	Edit colormap

Table 1: Overview of All Functions Present in the GUI Windows #1 and #2.

Once this is accomplished, the program creates a convex hull and automatically tracks the tip throughout the movie. Parameters extracted from the movie, such as a ratiometric kymograph, growth velocity, and filopodial length are displayed and also stored in the work folder as images and as data files. Other parameters such as filopodial lifetime, growth rate and retraction rate can then be extracted and further analyzed from the stored data files (**Figure 2B**).

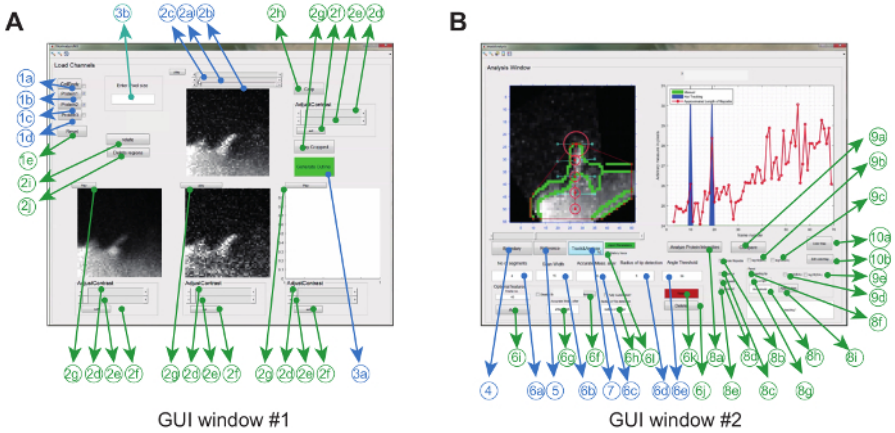

Figure 2: Graphical User Interface for using the Image Analysis Software. (**A**) GUI Window #1 is used for loading and processing images. The program can load up to 3 protein channels, whereby 2 channels are compared pair-wise. The window comes with mandatory (blue) and optional features (green) for pre-processing the images prior to tracking (**B**) GUI Window #2 is used for tracking the filopodium as well as spatio-temporal and ratio-metric protein analysis. Again, optional features are marked in green. This figure has been modified from reference [12]. Please click here to view a larger version of this figure.

Here, we present a detailed protocol for sample preparation and software handling. We start with detailed instructions on culturing cells and acquiring movies optimized for image analysis. This section on data acquisition is followed by a detailed description for operating the image analysis software. Throughout the protocol, we introduce critical steps and optional features that should be considered when collecting and processing data. Finally, we analyze filopodia from different model systems with the image analysis software, before closing with a comparison of the described image analysis software with other programs available for filopodial quantification and a discussion on limitations and future direction.

Protocol

1. Cell Culture

1. Culture HeLa or COS cells in Dulbecco's Modified Eagle Medium (DMEM) containing 4.5 g/L D-glucose, L-alanine-L-glutamine dipeptide, pyruvate, 10% fetal bovine serum, and 10 units/ml of penicillin/streptomycin. Culture neurons in culture-media without L-glutamine, glutamic

acid or aspartic acid, supplemented with 0.5 mM L-alanine-L-glutamine dipeptide, serum-free neuronal supplements and 10 units/ml of penicillin/streptomycin.
2. Once 40% confluency is reached, transfect cells with constructs of choice using a transfection reagent as per manufacturer's instructions. Keep transfected cells in incubator at 37 °C and 5% CO_2 for 15-18 h.
3. To reduce changes in pH and osmolarity (due to evaporation) during image acquisition, fill the cell culture chambers (prior to imaging) up to 90 % with 37 °C pre-warmed medium containing 20 mM 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (HEPES). To seal the lid, apply a thin layer of vacuum grease on the inside of the lid and gently press it on the culture chamber containing the transfected cells.

2. Image Acquisition

NOTE: The length of filopodia varies from 2-10 µm [13]. Filopodia grow at an average velocity of 0.05-0.1 µm/s [13,14].

1. Acquire images using a microscope with 60X or 100X objective and no pixel binning (here, a spinning disc confocal microscope). Use acquisition-rates greater than 1 Hertz (Hz) to track filopodial dynamics. To minimize out-of-focus artifacts, image filopodia close to the basal membrane (*i.e.* substrate surface).
2. To assure smooth tracking, adjust the exposure time of the camera and laser intensity such that the signal to noise ratio (SNR) is greater than 4. Avoid saturation of individual channels (*i.e.* pixel values of 255 for 8-bit images and 65,535 for 16-bit images), as this will preclude subsequent image analysis.
3. To avoid bleed-through, only use fluorescence tags compatible with laser lines and filters of the microscope (for details see reference [15]).

3. Image Pre-processing

NOTE: Use ImageJ or other available software to pre-process images [16,17].

1. If the sample is moving, correct for lateral drift using available software (*e.g.* https://github.com/NMSchneider/fixTranslation-Macro_for_ImageJ) prior to analysis. Exclude movies with axial drift (i.e. movement in z-direction).
2. Correct background (*i.e.* grey values of areas outside of the cell), bleaching (*i.e.* continuous loss if fluorescence intensity due to damage to fluorescence proteins) and possible bleed-trough (*i.e.* signal from one fluorescence probe in the both channels) using available software (*e.g.* http://imagej.net/Category:Plugins and [16,17]).
   Note: Fluorescence intensities of individual channels are not altered by the software.
3. To ensure subsequent image analysis, save movies corresponding to particular protein channels in grey scale as '.tiff' stacked file format in the work folder.
   NOTE: The dimensions (*i.e.* size, length) of the stacks must be same for all channels.

4. Image Analysis – Step 1: Load Images

NOTE: The software described here was written in Matlab (referred to as programming software) and will run only with this program.

1. Download the zipped folder containing all required files for image analysis from the following site: https://campus.uni-muenster.de/en/einrichtungen/impb0/nanoscale-forces-in-cells/software/. Unzip and copy files into the work folder.
2. Once installed, open the programming software and run 'filopodiaAnalysisM3.fig'. GUI Window #1 will open as shown in **Figure 2A**.
3. Load the saved stacked '.tiff' files corresponding to a particular protein of interest in GUI Window #1 using the buttons <1b> for protein A, <1c> for protein B, <1d> for protein C. See **Figure 2** and **Table 1** for details.
   NOTE: Protein A shown in GUI Window #1 acts as the reference channel for the final ratiometric analysis.
4. Create a superimposed image of the cell from the protein channels by clicking <1a>.
5. Click on <2a> to assign the first frame and <2b> for the last frame used for analysis.
6. Optionally, crop the region of interest (ROI) containing the filopodium of interest using button <2h>, rotate the image using button <2i>, or delete unwanted regions using the free-hand drawing tool <2j>.
   NOTE: To streamline image analysis, it is recommended to isolate the ROI (*i.e.* crop, rotate, delete) together with the other preprocessing steps (background subtraction, bleach corrections, etc.) using ImageJ.
7. Move the slider <2c> for each frame for quality control and to check whether the filopodium remains clearly visible throughout the whole movie.

5. Image Analysis – Step 2: Generate Trace

1. Click on button <3a> in the GUI Window #1 to open GUI Window #2 (see **Figure 2B**).
2. Click on button <4> in the GUI Window #2 to generate the mask of the superimposed cell body (generated in GUI Window #1 after clicking <1a>). The program also generates the boundary of the mask, where the convex hull is implemented to get the vertex points.
3. Click button <5>; a cursor will appear. Use the cursor to select the base (from where the distance of the filopodial tip is measured) followed by the tip of the filopodia in the frame where it first appears. To do this, move the slider in Window #2).
   NOTE: To minimize errors in data output, place the base point vertically below the filopodial tip along the axis. Positioning the base point on other places (*e.g.* laterally shifted) may introduce a directional bias in fluorescence values within the cell body.
4. Select the threshold length (above which the filopodia will bend) using <6c>.
   Note: This length is defined as the distance between the base point (selected using <5>) and the boundary of the cell body (*i.e.* the region from where the filopodia starts growing). The distance is measured is in pixels.
5. Specify the number of segments used to approximate the shape of the filopodia in box <6a>.
   NOTE: The minimum number of segments depends on the maximal length reached by the filopodium, but also how the filopodium bends. Do not select a number of segments larger than the number of pixel between the base and the tip (*i.e.* the threshold length). Selecting more

segments than the threshold length defined in step 5.4. (*i.e.* number of pixels between base and tip) will result in an overestimation of the length of the filopodium.

6. Specify the scan width, which acts as horizontal scanner to place the nodes, in <6b>.
   NOTE: These nodes are used by the program to fit the line joining the base and the tip with the body of the filopodium (*i.e.* to create the backbone). As a starting point, put a value in pixels equal to the maximum length multiplied by a factor of $\frac{1}{\sqrt{2}}$.
7. Specify the scan radius (in pixels) in box <6d>. Use a value that is approximately 50% larger than the observed inter-frame tip displacement.
   NOTE: Using a very large scan radius might grab unwanted convex-hull points in frames where no real filopodial tip is present (*e.g.* out of plane movement or low SNR).
8. Specify the bending angle in box <6e>.
   NOTE: The angle threshold is determined by the maximum angle the filopodium is bending during the whole analysis. Specifying the angle threshold helps the software to exclude tracking of unwanted structures growing from the side of the filopodia when the filopodia bends towards the cell body. The program works reliably for filopodia with tilting angles less than 45 degrees from the vertical axis.
9. To start tracking, click the <Track&Analyze> button in GUI Window #2. Click the 'History trace' box in GUI Window #2 to save the whole tracking protocol for future reference.
   NOTE: After the tracking procedure is complete, the length of the filopodium in each frame is stored in the sheet named 'length_vel' of 'dynamics.xlsx' for future references. Likewise, all other tracking parameters are stored in the sheet named 'parameters' of 'dynamics.xlsx'.
10. **Optionally, if the filopodia is not automatically detected in all frames, use the following steps to manually correct for it.**
    1. Visit the respective frame using the slider in Window #2 and manually select the tip of the filopodium.
       Note: Frames, where no convex hull points are detected will be represented by a blue region in the tracking window of GUI Window #2. It is mandatory to check the 'History trace' button before the tracking program was initiated for accessing the nodal coordinates in that frame.
    2. Select the reference points (base followed by tip) using <6f> in GUI Window #2. Specify the other parameters such as 'scan length', 'Accurate Measurement after', and 'maximum bending angle' for that frame as described in steps 5.4-5.8.
    3. Click button <6i> to save the new parameters (specific to that one frame). The steps are to be repeated for all frames indicated by the blue region.
    4. When finished, re-initialize the tracking procedure using <7>.
       NOTE: This correction can also be used if the program suddenly switches from one filopodial to another within a movie. As an alternative, consider cropping movies that contain multiple filopodia.

6. Image Analysis – Step 3: Spatio-temporal Protein Analysis

1. For spatio-temporal analysis, select the box represented by <8b> followed by the protein channel of interest (<8c> and/or <8d> and/or <8e>).
   Note: It is mandatory to select Protein A prior to ratiometric analysis (<9a>, <9b> and/or <9c>) since Protein A is used as reference.
2. Click on <8a> to initiate protein tracking along the filopodial length using the trace generated in step 5.9.

7. Image Analysis – Step 4: Ratio-metric Protein Analysis

1. Check box <9b> or <9c> and click on the button <9a> to get the spatio-temporal ratiometric plot.
   NOTE: To avoid misrepresentation of relative protein concentration, the ratiometric image is not plotted as X/Y but as log(X/Y). For future use, the ratiometric plot is exported in '.png' and '.fig' format files, and raw plot data is stored within the 'dynamics.xlsx' file.

8. Image Analysis – Step 5: Filopodial Tip Analysis

1. Check box <8f> and specify the tip length and threshold length from the base using <8g> and <8h>. Click on <push button> to save data to file 'dynamics.xlsx'. Click on <Analyze Protein Intensities> to generate the trace of protein intensities of the filopodial tip and to save for ratiometric analysis.
   NOTE: Tip length determines the number of pixels at the tip used for the analysis. The software will return the average intensity value of the pixels at the tip over each frame. The threshold length determines the minimum distance from the base to tip above which the filopodium (filopodial tip) starts growing (and when the program starts tracking the intensity value of the tip). So the tip length must be smaller than the threshold length.
2. Click on the desired ratio to be analyzed using <9d> or <9e>.
3. Click on the compare button <9a> to generate the ratiometric data.

Representative Results

Using COS cells transfected with a marker for filamentous actin (f-tractin[18], red) and a cytosolic reference (green), we found actin-rich filopodial protrusions (**Figure 3A**, top panel). Time series showed that filopodia rapidly extend and retract (**Figure 3A**, middle panel). Using the image analysis software, we then traced individual filopodia. Comparison of filopodial length measured by hand vs. the image analysis software showed a Pearson cross-correlation value of 0.947 arguing that the software reliably tracked filopodial extension [12]. In addition to growth and retraction rates of an individual filopodia, kymographs at the bottom of **Figure 3A** also depicts fluorescence intensities of actin (top), the cytosolic reference (middle), and the relative concentration of actin normalized to the cytosolic reference (bottom). Together, these experiments provide evidence that the program reliably measures growth dynamics as well as spatially resolved relative protein concentration throughout the full life-cycle of an individual filopodium in COS cells.

Next, we investigated filopodia dynamics in cultured mouse hippocampal neurons (**Figure 3B**, top panel). For this, neurons were transfected at day *in vitro* (DIV) 8 with f-tractin (red) and a cytosolic reference (green) (**Figure 3B**, middle panel). As in the previous experiment, kymographs depicting fluorescence intensities of actin and cytosolic reference showed that relative enrichment of actin preceded formation of exploratory dendritic filopodia (**Figure 3B**, bottom panel). These findings are in line with published work, describing formation of actin-rich patches prior to filopodial elongation [3,19,20].

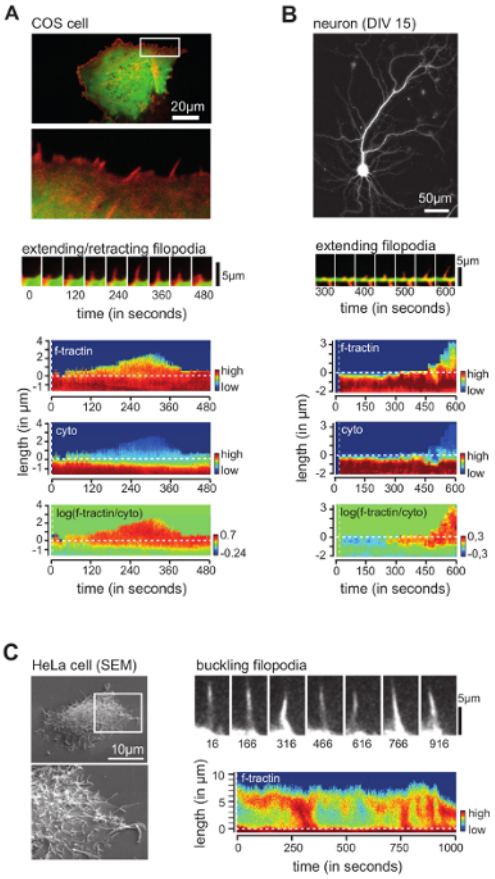

Figure 3: Examples of Filopodia Analyzed by the Software. (**A**) Overview image (top panel) and time series (middle panel) of COS cells transfected with f-tractin (red) and cytosolic marker (green). Below, plots depicting analysis of filopodial growth dynamics and relative concentration of f-tractin along the filopodium (bottom panels) are shown. (**B**) Overview image of hippocampal neuron transfected with cytosolic marker (top) and time series of neuron transfected with f-tractin (red) and cytosolic marker (green) (middle panel), as well as plot depicting analysis of dendritic filopodial growth dynamics and relative concentration of f-tractin along the protrusion (bottom panels). (**C**) SEM image (left panel) and time series of HeLa cells transfected with f-tractin (right panel). Note fluctuations in fluorescence intensity due to filopodial buckling. Scale bars = 20 µm (A, top), 50 µm (B, top), 10 µm (C, top). This figure has been modified from reference [12]. Please click here to view a larger version of this figure.

Finally, we aimed to challenge the software by monitoring filopodia in HeLa cells. Filopodia in HeLa cells are long (**Figure 3C**, left panel) and highly dynamic, often leaving the plane of acquisition. Intriguingly, when focusing on the basal membrane, we observed a sub-fraction of filopodia to undergo helical twisting (**Figure 3C**, top right panel), whereby parts of the filopodium transiently left the focal plane. Accordingly, kymographs depicting the absolute fluorescence intensity of f-tractin showed wave-like changes in fluorescence intensity through time (**Figure 3C**, bottom right panel). This behavior is reminiscent of filopodial buckling, a mechanism recently described to be used by filopodia to exert pulling forces [5].

In summary, these experiments provide evidence that the software reliably tracks protein concentration and growth dynamics of filopodial from various origins.

Discussion

Here we present a detailed protocol for tracking filopodial growth dynamics and analysis of relative protein concentrations in these dynamic structures via the convex-hull algorithm. Using the software, up to 3 channels can be compared pair-wise in a single run, whereby the relative concentrations of two channels (*i.e.* proteins) is determined throughout the extension/retraction cycle and stored as image and data files in separate folders. In addition to the routine operations, the software also provides a number of parameters that can be modified to optimize analysis for the respective experiment. A detailed description of these modifications as well as troubleshooting can be found in the protocol section and in **Table 1**.

Image analysis is not limited to a particular microscope type, but rather by the image quality of the time series. Considering its relevance, image quality should therefore be optimized for each channel during acquisition. Our experiments show that the algorithm works best for filopodia with SNR >4 acquired at 1 Hz using an objective with magnification above 40X and no pixel binning. Of course, this applies only as long as the fluorophores are compatible with the filter settings (*i.e.* no bleed-through; see protocol section 2 for details). Considering that relative intensities are compared against each other, the analysis is not sensitive to small differences in absolute fluorescence signal intensities between individual cells. Therefore, to deduce meaningful data, a well-defined reference channel (*e.g.* cytosolic marker) is of great importance. However, even if image quality is good there are some biological limitations. For instance, it is not well suited to analyze structures that are branching, tilting more than 45 degree from the axis orthogonal to the cell surface, or where other objects are crossing the structure that is being investigated.

In addition to these experimental limitations, there are critical steps within the protocol that need to be considered when operating the software, as this may otherwise trigger error messages. First, channels corresponding to a particular protein must be of same dimension and represent exactly the same ROI. Second, the stacked files must be in gray scale '.tiff' file format. Third, the stacked image files must be in the work folder. And finally, the names for data files in the work folder should not be changed, unless this is corrected for in the code.

How does the script compare to other existing methods? To probe our image analysis software, we downloaded a number of recently published software solutions that analyze filopodial features [6,10,21,22,23,24,25], and tested all for a number of selected criteria. A detailed comparison is presented in **Table 2**.

	Tsygankov *et al.*	**Barry *et al.***	**Nilufar *et al.***	**Fanti *et al.***	**Constantino *et al.***	**Hendricusdottir *et al.***	**Tarnok *et al.***	**Saha *et al.***
Paper DOI	10.1083/jcb.201306067	10.1083/jcb.201501081	10.1186/1752-0509-7-66	10.1002/dneu.20866	10.1016/j.jneumeth.2008.02.009	10.1016/j.jneumeth.2014.08.016	10.1002/cyto.a.22569	10.1091/mbc.E16-06-0406
software (download link)	http://www.hahnlab.com/tools/THE softwarepage.html	http://jcb-dataviewer.rupress.org/jcb/browse/9059/	http://www.perkinslab.ca/pubs/NMLP20XX.html	http://www.ifc.unam.mx/ffm/download.html	http://fournierlab.mcgill.ca/styled-7/fTracker.html	https://fdynamics.wordpress.com/	http://cnblab.elte.hu/dfma	https://campus.uni-muenster.de/index.php?id=13794&L=1
program type	Matlab	ImageJ	Matlab	ImageJ	Matlab	Matlab	ImageJ	Matlab
operation	automated	automated	automated	semi-automated	semi-automated	semi-automated	semi-automated	semi-automated
single vs. batch analysis	both	both	single	single	single	single	single	single
whole cell vs. subregion analysis	whole	whole	both	both	both	sub	sub	sub
multiple vs. individual filopodia	multiple	multiple	multiple	multiple	multiple	ind	ind	ind
filopodial ID and visual inspection possible	yes	yes	no	yes	yes	yes	yes	yes
Image processing possible	no	no	no	yes	no	no	no	yes
filopodial length	yes	yes	yes	yes	yes	yes	yes	yes
filopodial growth dynamics	yes	yes	no	yes	yes	yes	yes	yes
filopodial lifetime	yes	yes	no	yes	yes	yes	yes	yes
ratiometric analysis in filopodia	no	no	no	no	no	no	no	yes
output / storage format	images / data file	images / data file	images / data file	images / data file	images / data file	images / data file	images / data file	images / data file

Table 2: Overview of Software-assisted Image Analysis Solutions for Filopodia Quantification.

From the eight tested analysis software solutions, five were operated *via* Matlab, while the remaining three used ImageJ. Two software solutions (**Table 2**, to the left) were fully automated and optimized for batch analysis of filopodia length, lifetime and dynamics in whole cells. Of the remaining six software solutions, five required additional manual input (*i.e.* semi-automated), while one was fully automated. However, only the semi-automated solutions provide detailed information on filopodia growth dynamics, life time and identity. Among these remaining five, three were optimized for subcellular analysis of individual filopodia, while two could process multiple filopodia in parallel. Importantly, from all eight tested software solutions, only one approach allowed to combine information on filopodial growth dynamics with ratiometric protein localization within these finger-like structures (**Table 2**, to the right).

Although our image analysis software is not suitable for automated batch or whole cell analysis, quantification of an isolated filopodia takes only minutes. Thus, we envision that quantification of protein dynamics in 40-50 filopodia is feasible within a single day with the current software. In contrast, high-throughput screens may require further amendments to the code to streamline input and storage of thousands of individual filopodia in an automated manner. Based on the parameters that are required, other existing software solutions may thus be better suited for such an approach. Keeping these limitations in mind, the software provides a reliable platform for investigating spatio-temporal protein concentration in protrusion like structures in cells. Considering that filopodia are not the only fingerlike cellular structures, it is plausible to envision that the presented image analysis software may also be suitable to study protein dynamics in other biological processes (*e.g.* neurites, primary cilia).

Disclosures

The authors have nothing to disclose.

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