

Video Article

Using plusTipTracker Software to Measure Microtubule Dynamics in *Xenopus laevis* Growth Cones

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Abstract

Microtubule (MT) plus-end-tracking proteins (+TIPs) localize to the growing plus-ends of MTs and regulate MT dynamics^{1,2}. One of the most well-known and widely-utilized +TIPs for analyzing MT dynamics is the End-Binding protein, EB1, which binds all growing MT plus-ends, and thus, is a marker for MT polymerization¹. Many studies of EB1 behavior within growth cones have used time-consuming and biased computer-assisted, hand-tracking methods to analyze individual MTs¹⁻³. Our approach is to quantify global parameters of MT dynamics using the software package, plusTipTracker⁴, following the acquisition of high-resolution, live images of tagged EB1 in cultured embryonic growth cones⁵. This software is a MATLAB-based, open-source, user-friendly package that combines automated detection, tracking, visualization, and analysis for movies of fluorescently-labeled +TIPs. Here, we present the protocol for using plusTipTracker for the analysis of fluorescently-labeled +TIP comets in cultured *Xenopus laevis* growth cones. However, this software can also be used to characterize MT dynamics in various cell types⁶⁻⁸.

Video Link

The video component of this article can be found at <http://www.jove.com/video/52138/>

Introduction

The goal of this method is to obtain quantitative information regarding microtubule (MT) plus-end-tracking protein (+TIP) dynamics in living growth cones. MT +TIPs are a group of proteins that localize to the plus-ends of MTs^{9,10}. They perform a range of functions to regulate parameters of MT dynamic instability¹¹, including rates of polymerization, catastrophe, and rescue. One well-used method for analyzing MT dynamics is to track the behavior of the +TIP EB1, which binds specifically to growing MT plus-ends^{1,12}. EB1 is known to recruit several other proteins to growing MT plus-ends^{13,14}, and has recently been established as a MT maturation factor¹⁵, promoting both MT growth and catastrophe frequency^{15,16}.

Many studies of MT dynamics within growth cones have utilized hand-tracking methods to measure changes in EB1-GFP dynamics over time¹⁻³, as EB1 localization to MT plus-ends can be used as a marker for MT polymerization. A key benefit for examining EB1-GFP comets as a proxy for MT growth is that MT dynamics can be measured even in regions of significant MT overlap. While the method of hand-tracking EB1-GFP comets has provided useful insights into MT behaviors¹⁻³, it is time-consuming and can be biased. Additionally, as aberrant growth cone behaviors are likely the result of minute shifts in cytoskeletal dynamics, analyzing only a small subset of MTs (usually necessary when hand-tracking) may miss significant information.

Thus, we measure global MT dynamics parameters using the software package, plusTipTracker⁴, after the acquisition of high-resolution, live images of tagged EB1 in cultured embryonic growth cones⁵. This software, developed in the Danuser Lab, has been used in several studies characterizing MT dynamics in various cell types⁶⁻⁸. It is an open-source, user-friendly, MATLAB-based package that includes automated detection, tracking, visualization, and analysis for movies of fluorescently-labeled +TIPs. A long list of specific parameters of MT dynamics are calculated by this software (see Reference 4 for details), but for analysis of MT dynamics in growth cones, the most useful parameters are MT growth track velocity (in microns/minute), growth track lifetime (in seconds), and growth track length (in microns). The software can be downloaded directly from the Danuser Lab website (under "Software"). While the Danuser Lab currently supports a newer interface for +TIP tracking analysis, which is incorporated into a software package called u-track 2.0, the original, stand-alone software will remain available. The underlying algorithms between the two programs are the same (at least as of 2014), with only a difference of interface and analysis outputs. For the novice user with little MATLAB and/or computational analysis experience, plusTipTracker has more user-friendly features, including automated statistical parameter outputs.

Here, we describe the steps for analyzing images of EB1-GFP dynamics in cultured *Xenopus laevis* growth cones. This protocol was utilized in a recent paper examining MT dynamics¹⁷. See also Lowery *et al.* 2012⁵ for detailed instructions regarding culturing growth cones expressing EB1-GFP. While this paper primarily focused on examining EB1-GFP dynamics in growth cones, the same protocol can be used for other cell types¹⁷.

For all cell types, the time interval between frames should be between 0.5-2 sec for optimal +TIP tracking. A time interval of up to 4 seconds between frames is possible, but this increased interval time results in additional tracking errors.

Protocol

This protocol and video are meant to serve as a companion to the original paper describing the software package in more detail⁴, as well as the Technical Report that comes with the software download on the Danuser Lab website. Readers are encouraged to review these documents carefully if there are additional questions regarding using the software.

1. Prior to Image Analysis

1. Convert each time-lapse movie into a sequence of TIFF (Tagged Image File Format) image files. If there are multiple growth cones/cells in a given movie, first crop each growth cone/cell to create its own image sequence.
Note: This is not necessary, as individual regions-of-interest (ROI) can be selected within plusTipTracker. However, using smaller image dimensions increases the speed of the computational processing, so this step is recommended if there is significant blank space in the image.
2. Save each TIFF series in its own folder called "images" within a path that MATLAB is set to access (note that "images" is case-sensitive). To add a new path, navigate to the relevant file directory in the "Current Folder" window, right-click on the directory icon, and select "Add to Path – Selected Folders and Sub-Folders". It is important that the plusTipTracker software folder be added to the Path, as well.

2. plusTipGetTracks

Note: The first step in image analysis is to detect the EB1-GFP comets, link the comets into tracks, and determine the parameters of microtubule dynamics. This is obtained with the command "plusTipGetTracks"⁴.

1. To begin analysis, open MATLAB application and type "plusTipGetTracks" into the command window. This will cause a new dialog box to appear.
2. Click on "Set Up New Projects" and select one (or more) of the previous TIFF image series by selecting the appropriate "images" folder (or directories containing "images" folders). Upon completion of this step, a file directory (roi_1) will be created (in the same folder that holds "images") that will contain the future data files. Note: the "Set Up New Projects" step can be completed ahead of time, during a separate session.
3. A new window will appear: "Select a polygon, right-click on last point, and click on 'Create Mask'". Click on "OK". The first image of the selected image series will then be displayed. Use the mouse to click and create a polygon that encompasses the entirety of the growth cone. Double click the mouse to close the polygon.
4. Once the polygon has been closed, a dialog box will appear: "Do you want to select another ROI?" If the image has another growth cone to analyze, select "Yes"; otherwise select "No".
5. Select the projects that will be immediately analyzed. Click on "Select Projects" and select the folder (roi_X) to analyze.
6. A listSelectGUI screen will appear. Select the project(s) from the left side of the screen and move them over to the right side of the screen. Click on "OK". Choose a location to save the project list and click on "Save".
7. Select "Detection", "Tracking", and "Post-Processing". Once these selections have been made, the right side of the dialog box will become configurable. Configure each option.
 1. These parameters are used to link detected comets into MT tracks. Details for choosing these control parameters for tracking are included on pages 9-10 of the Technical Report PDF which accompanies the software package download; read this report carefully if problems are encountered. For the purposes of tracking EB1-GFP comets in *Xenopus laevis* growth cones, use the following Tracking Settings: Search Radius Range (pixels) 5-12, Minimum Sub-Track Length (frames) 3; Max Gap Length (frames) 8; Max Shrinkage Factor 0.8, Max angle Forward 50, Max angle Backward 10, Fluctuation Radius 2.5. These settings are shown in **Figure 1**.

Note: Max Shrinkage Factor is set to reduce the number of "backward gaps" detected, as "backward gaps" are not useful to analyze in the context of growth cones, given the crowded conditions and likely errors in track linkages. Additionally, both Max Forward angle as well as Fluctuation Radius are set relatively high, as growth cone MTs exhibit small frequent translocations in addition to growths and shrinkages, and increasing these control settings allows for this increased movement during the linkage step.

1. Fill in the Post-Processing Settings depending upon the desired specific image acquisition settings.
8. Once the settings have been configured, click on "Start". The software will run whichever settings have been selected. This may take minutes to hours, depending on the number of projects selected and their sizes. The Command Window displays the estimated time remaining for each function. When the plusTipGetTracks step is completed, the Command Window will display "Finished!"
Note: A long list of specific parameters of MT dynamics have now been calculated by this software (see Reference 4 for details), but for analysis of MT dynamics in growth cones, the most useful parameters to examine are MT growth track velocity (in microns/minute), growth track lifetime (in seconds), and growth track length (in microns).

3. plusTipSeeTracks

Note: Now that the microtubule tracks have been defined, the function "plustipSeeTracks" is used for track visualization⁴. This function can provide multiple outputs for visualization, including spatial MT dynamics maps and speed movies, but here, the focus is solely on using "Track Movies" to display MT tracks superimposed on the growth cone images. While plusTipGetTracks can analyze multiple movies at a time, plusTipSeeTracks can only analyze one movie at a time.

1. Type "plusTipSeeTracks" into the command window.

2. After the dialog box loads, click on "Select Project". Select the parent directory containing the project to visualize and click on "Select Folder". A new window will appear: "Select the project you want to visualize". Choose the file to visualize and click on "OK".
3. Next, click on "Select Saved ROI". Navigate to the same roi_X folder as the one selected in the previous step and select the file named "roiYX".
4. Click on "Select Output Directory" to designate where MATLAB will save the track visualization files. Note: We recommend using the same folder that contains the rest of the data.
5. Select "Make Track Movie" and a screen will appear displaying all of the tracks plusTipGetTracks calculated from the +TIP comets. This step saves the tracked time series in a movie format, in the file "allTracks_X_X_X". There is an option for saving the movie as an AVI, otherwise the default format is as a Quicktime.mov file.

4. plusTipGroupAnalysis

Note: This final function is used to create groups of movies for analysis and comparison of their MT track parameters.

1. Type "plusTipGroupAnalysis" into the command window. To manually select the groups to compare, first de-select "Auto group from hierarchy". Then, click on "Select projects". Navigate to the parent directories containing all the roi_X folders to analyze.
2. A listSelectGUI screen will appear. Select all of the projects to include in the groups from the left side of the screen and move them over to the right side of the screen. Click on "OK". Choose a location to save the project list and click on "Save".
3. A window will appear: "Please select first group from the list". Click on "OK". The listSelectGUI window will display again. This time, select only those files that correspond to the first group that should be pooled together. Click on "OK".
4. Then, enter the group name, and click on "OK". A window will appear: "Select another group?" Answer accordingly and continue selecting groups. A window will appear: "Select a location to save your group list". Navigate to the location and click on "Save".
5. Click on "Select Output Directory" to choose where the output folders will be stored.
6. Select which type of group analysis to conduct – whether the MT tracks should be pooled for each group or per cell analysis should be performed. The recommended statistical tests are already designated. To include all of the tracks in the analysis, de-select "Remove tracks at beginning/end of movie". Otherwise, having this box selected removes any MT growth tracks that are in process as the movie begins or ends.
7. After the Group Analysis selection is made, select "Compare groups".

Representative Results

Using this software as described here will provide several files of information that quantify +TIP dynamics in living cells.

The function plusTipGetTracks identifies the tracks (using example settings shown in **Figure 1**), and then provides parameters regarding the +TIP tracks. To view the information that the software has obtained, go into the roi_X directory that was created in step 2.2. The "feat" folder contains "overlayImages", which is a series of images showing the detected comets. Examining these images using image analysis software can demonstrate the accuracy of comet detection. The "meta" folder also contains detailed information regarding +TIP comet statistics, including the "projData" file, as well as the "Stats" file. To view the "Stats" file, drag it into an open worksheet of a spreadsheet application. This file contains the calculated microtubule parameters for each movie (**Figure 2**). As noted above, a long list of specific parameters of MT dynamics are calculated by this software (see Reference 4 for details), including MT growth track velocity (in microns/minute), growth track lifetime (in seconds), and growth track length (in microns).

The function plusTipSeeTracks saves a movie of the tracked comets, which can be reviewed by opening the file "allTracks_X_X_X" (**Figure 3**).

The function plusTipGroupAnalysis combines multiple individual data sets into groups and creates folders (named perCell or pooledData, depending on which analysis is selected) that contain group parameter data, including histograms, plots, and spreadsheets for comparing groups and individual parameters within each group (**Figure 4**).

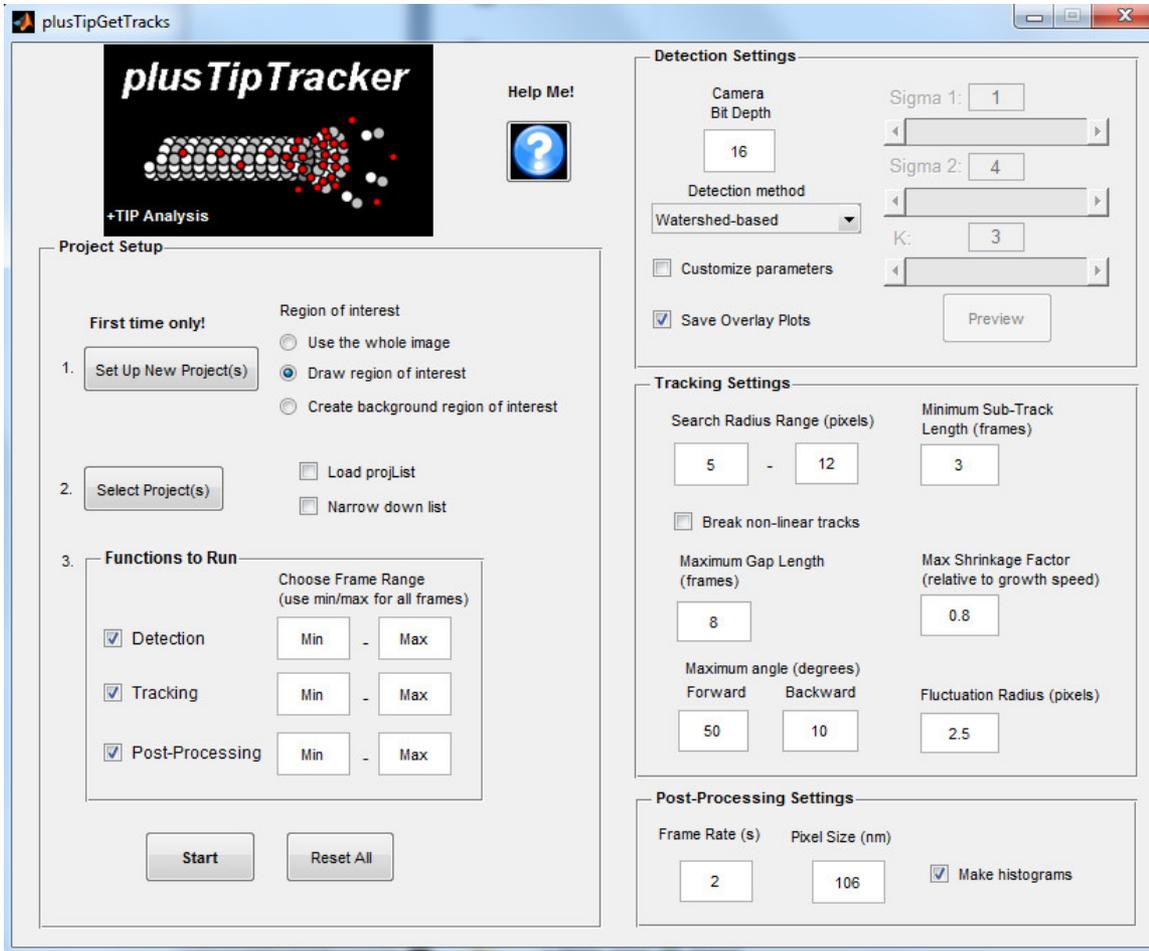


Figure 1. PlusTipGetTracks settings used for EB1-GFP comets in *Xenopus laevis* growth cones. This figure shows the specific settings for “Detection”, “Tracking”, and “Post-Processing” steps that may be used for the analysis of EB1-GFP comets in *Xenopus laevis* growth cones. The plusTipParamSweepGUI tool, available within the plusTipTracker package, may be used to optimize tracking settings for other model organisms and/or cell types⁷.

	A	B	C	D
1	medNNDistWithinFrameMic	1.51123		
2	nGrowths	22		
3	growth_speed_median	5.65202		
4	growth_speed_mean	6.43951		
5	growth_speed_std	2.44565		
6	growth_lifetime_median	16		
7	growth_lifetime_mean	16.1818		
8	growth_lifetime_std	11.6277		
9	growth_length_median	1.29563		
10	growth_length_mean	1.74266		
11	growth_length_std	1.44463		
12	nFgaps	0		
13	fgap_speed_median	NaN		
14	fgap_speed_mean	NaN		
15	fgap_speed_std	NaN		
16	fgap_lifetime_median	NaN		
17	fgap_lifetime_mean	NaN		
18	fgap_lifetime_std	NaN		
19	fgap_length_median	NaN		
20	fgap_length_mean	NaN		
21	fgap_length_std	NaN		
22	fgap_freq_time_mean	NaN		
23	fgap_freq_length_mean	NaN		
24	fgap_freq_time_SE	NaN		
25	fgap_freq_length_SE	NaN		
26	GrowthSpeedBeforeFgap_MicPerMin_mean	NaN		
27	GrowthSpeedBeforeFgap_MicPerMin_SE	NaN		
28	GrowthLifetimeBeforeFgap_Sec_mean	NaN		
29	GrowthLifetimeBeforeFgap_Sec_SE	NaN		
30	GrowthLengthBeforeFgap_Mic_mean	NaN		
31	GrowthLengthBeforeFgap_Mic_SE	NaN		
32	nBgaps	0		
33	bgap_speed_median	NaN		
34	bgap_speed_mean	NaN		
35	bgap_speed_std	NaN		
36	bgap_lifetime_median	NaN		
37	bgap_lifetime_mean	NaN		
38	bgap_lifetime_std	NaN		
39	bgap_length_median	NaN		
40	bgap_length_mean	NaN		
41	bgap_length_std	NaN		

Figure 2. Screenshot of MT parameters obtained from plusTipGetTracks analysis. The “meta” folder, created by running plusTipGetTracks, contains information regarding +TIP comet statistics. By dragging the “Stats” file into a spreadsheet application, microtubule dynamics parameters can be examined.

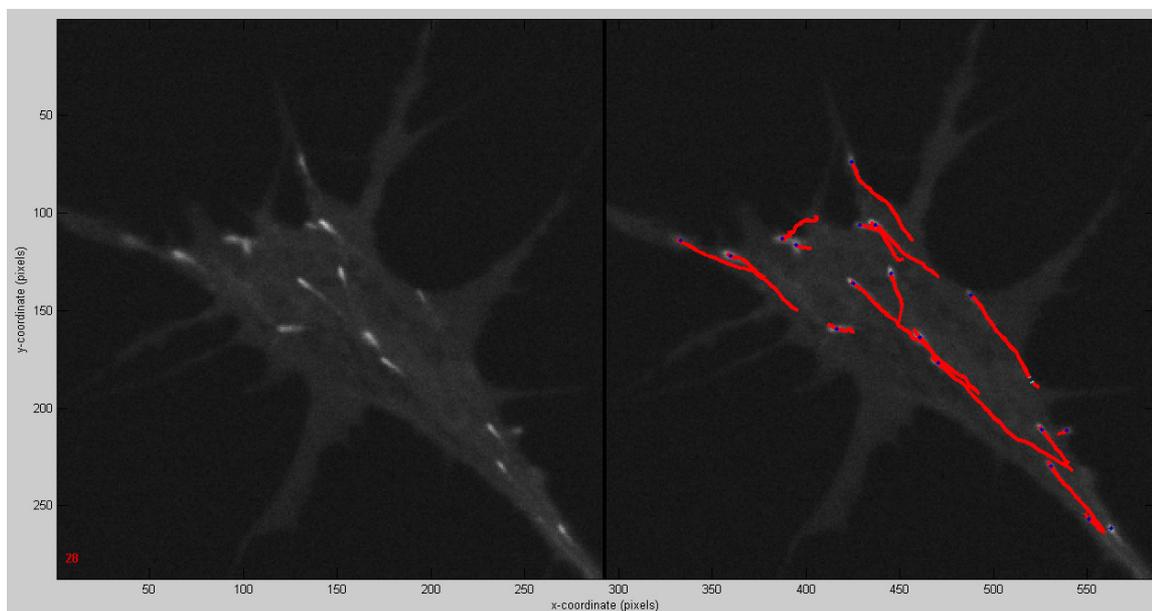


Figure 3. Screenshot of MT track movie obtained from plusTipSeeTracks analysis. PlusTipSeeTracks not only allows for microtubule track visualization, but also serves as a verification tool by allowing the user to view the validity of the data acquired from plusTipGetTracks.

	A	B	C	D	E
1		grp_control	grp_mutant		
2	medNNdistWithinFrameMic	1.09609	1.33852		
3	nGrowths	130	52		
4	growth_speed_median	7.61519	7.06354		
5	growth_speed_mean	8.66502	7.5267		
6	growth_speed_std	5.1866	3.6716		
7	growth_lifetime_median	8	9		
8	growth_lifetime_mean	10.0923	12.6538		
9	growth_lifetime_std	7.78733	10.0252		
10	growth_length_median	1.03707	1.02397		
11	growth_length_mean	1.45185	1.59862		
12	growth_length_std	1.29349	1.37517		
13	nFgaps	11	1		
14	fgap_speed_median	-0.490645	1.58326		
15	fgap_speed_mean	-0.931854	1.58326		
16	fgap_speed_std	3.20703	0		
17	fgap_lifetime_median	10	4		
18	fgap_lifetime_mean	10	4		
19	fgap_lifetime_std	3.2249	0		
20	fgap_length_median	-0.098129	0.10555		
21	fgap_length_mean	-0.101505	0.10555		
22	fgap_length_std	0.521414	0		
23	GrowthSpeedBeforeFgap_MicPerMin_mean	9.26559	10.5611		
24	GrowthSpeedBeforeFgap_MicPerMin_SE	1.3828	3.08845		
25	GrowthLifetimeBeforeFgap_Sec_mean	11.6	7		
26	GrowthLifetimeBeforeFgap_Sec_SE	1.65846	1		
27	GrowthLengthBeforeFgap_Mic_mean	1.82141	1.18065		
28	GrowthLengthBeforeFgap_Mic_SE	0.385802	0.184301		
29	fgap_freq_time_mean	0.121193	0.145833		
30	fgap_freq_time_SE	0.019694	0.0208333		
31	fgap_freq_length_mean	1.03329	0.868143		
32	fgap_freq_length_SE	0.212146	0.135518		
33	nBgaps	0	1		
34	bgap_speed_median	NaN	-0.462832		
35	bgap_speed_mean	NaN	-0.462832		
36	bgap_speed_std	NaN	0		
37	bgap_lifetime_median	NaN	4		
38	bgap_lifetime_mean	NaN	4		
39	bgap_lifetime_std	NaN	0		
40	bgap_length_median	NaN	-0.0308555		
41	bgap_length_mean	NaN	-0.0308555		

Figure 4. Screenshot of MT parameters obtained from plusTipGroupAnalysis. PlusTipGroupAnalysis offers the user a simple method for comparing groups and individual parameters between within each group by combining multiple individual data sets and generating statistical output, which can be examined in a spreadsheet application.

Discussion

PlusTipTracker provides a straightforward, graphical user interface to quickly and automatically detect virtually all visible EB1-GFP comets in a cell or growth cone, link the comets into tracks, and calculate MT parameters. Other publications have reported the design of similar types of software (for example, Marx *et al.* also utilized quantitative analysis of tagged EB1 dynamics in growth cones¹⁸). But, this software appears to be unique in its ease of access, as it is freely downloadable from the website of the Danuser Lab, which specializes in designing open-source, turn-key software useful to the cell biological community. While access to MATLAB is required, one does not need to be fully familiar with this computer application in order to utilize the software. However, there are a few points that need to be addressed for ease of use.

First of all, one of the most common issues that arise when using the software and computer application for the first time is related to the file path. If this error occurs (with the prompt "Error using cd – Argument must contain a string. Error in formatPath..."), then the easiest solution is to ensure that the plusTipTracker software, as well as the directory with all of the "images" subdirectories, are both in the same "MATLAB" file path. It is best if these are not in the "Program Files" directory, as it has been suggested that the space in the "Program Files" name may be an issue.

Related to this, it is important to note that plusTipTracker saves the file path that was utilized when first computing the plusTipGetTracks analysis, and as such, this file path must be maintained when this data is accessed and employed by another component of the software. The functions plusTipGetTracks, plusTipSeeTracks, and plusTipGroupAnalysis all use the original saved file path, and thus, trying to call those functions for a given movie, after moving the files to another path, will result in an error.

Another common error to occur during analysis is when Tracking fails during the plusTipGetTracks step. This will occur if a frame in the image series contains no detectable comets. This will completely halt the analysis and no post-processing will occur. An easy fix to circumvent this issue and allow the analysis to proceed, is to create a mock comet on the image in an area where it will not be incorrectly linked to any actual tracks. This will not impact the final track parameters, as any comet that does not exist in a minimum number of consecutive frames will be filtered out of the final analysis.

One other issue that may arise is faulty comet detection. This can usually be fixed by improving the region-of-interest selection in step 2.3. It is important to draw the region of interest closely around the cell and not to draw a wider region than is necessary. The software uses this region to determine the background used during comet detection. If comet detection is still sub-optimal with the default settings, the settings can be adjusted in the plusTipGetTracks window (during step 2.7).

After any analysis, it is critical to validate the automated track linkages by eye, using plusTipSeeTracks. Tracking settings may need to be modified to reduce the numbers of false positive or false negative comet linkages. See the original plusTipTracker documentation⁴ as well as the Technical Report PDF that accompanies the software download for details on optimizing the settings. The performance of this software compared to hand-tracking has previously been tested in non-neuronal cells⁴. Growth cones pose a slightly different challenge, however, as growth cone MTs exhibit frequent translocations in all directions¹⁷, in addition to MT growth and shrinkage. One issue that was not found to be a major concern is whether the tightly packed MTs in growth cones pose tracking difficulties¹⁷. As only a subset of MTs are in the growing phase, with EB1-GFP on the ends, resolving and tracking individual EB1-GFP comets was not problematic. However, it should be noted that these previous studies used *Xenopus laevis* growth cones, which were specifically chosen because of their relatively large growth cone size (approximately 10 microns), compared to other vertebrate growth cones. Using these larger growth cones allows for more accurate EB1-GFP comet analysis.

To assess the utility and accuracy of this software for analyzing EB1-GFP tracks in *Xenopus laevis* growth cones, we compared the experience of using plusTipTracker with hand-tracking of an identical data series (data not shown). The time it took to hand-track EB1-GFP comets in an average growth cone of 39 comet tracks (in a 1 minute time-lapse series, with 2 seconds between each frame) was over two hours, compared to two minutes with the software. The parameters obtained with the two methods were similar for MT growth velocity (7.4 microns per minute for automated tracking versus 7.0 microns per minute for hand-tracking). However, for growth lifetime and length, software analysis leads to significantly shorter tracks (by about half the time and distance). This is due to growth tracks being split by the software if a comet goes in and out of focus over time. While the human eye can easily identify that it is the same comet, the software does not. This issue is not problematic though, if one is using the software in order to compare multiple conditions. Since identical tracking parameters are used for all conditions (and assuming that comets go in and out of focus at the same rate in multiple conditions), then the relative lifetimes and lengths are still quite useful measurements for comparison. As for automated analysis error rates, these depend greatly on the quality of the images. In high signal-to-noise movies, the percent of misjoined or incorrect tracks is in the single digits. Even in lower-quality movies (where individual comets are still clearly visible by eye, but the background noise is greater), the error rates are still low enough (5-15%) that the significant time saved by using the software is worth the costs in error. This is especially the case when analyzing hundreds of growth cones (sixty to eight growth cones per condition were analyzed in a previous study¹⁷).

It is important to note that this software was designed for detecting +TIP comets that only bind to growing MT ends, such as EB1-GFP. Given that the linkage and tracking algorithms expect that comets only exist on polymerizing MTs, using this software to analyze the dynamics of a fluorescently-tagged +TIP that binds to shrinking MT ends in addition to growing ends will lead to incorrect information regarding calculated MT growth velocities.

One of the unique features of this software compared to other single-particle-tracking software, is that it takes into account known MT behaviors to calculate not only polymerization parameters, but also shrinkage parameters. It does this by linking an EB1-GFP comet that has disappeared with one that has newly formed directly behind it in the same trajectory (this is called a backgap, or bgap track). While this algorithm works well for some cell types, such as HeLa cells⁴, it is a less effective feature when analyzing MT dynamics in growth cones. This is because MT tracks frequently follow each other along the exact same paths in growth cones (often following along F-actin bundles), and so it is usually impossible to tell if bgap linkages are correct. For this reason, it is not recommended to utilize the bgap data outputs in growth cones.

Despite these minor caveats and issues which must be taken into consideration when using plusTipTracker (and most any automated image processing program), this software can be a very useful tool for analyzing thousands of EB1-GFP comets in a relatively short amount of time.

Disclosures

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