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plusTipTracker: quantitative image analysis software for the measurement of microtubule dynamics

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Abstract

Here we introduce plusTipTracker, a Matlab-based open source software package that combines automated tracking, data analysis, and visualization tools for movies of fluorescently-labeled microtubule (MT) plus end binding proteins (+TIPs). Although +TIPs mark only phases of MT growth, the plusTipTracker software allows inference of additional MT dynamics, including phases of pause and shrinkage, by linking collinear, sequential growth tracks. The algorithm underlying the reconstruction of full MT trajectories relies on the spatially and temporally global tracking framework described in (Jaqaman et al., 2008). Post-processing of track populations yields a wealth of quantitative phenotypic information about MT network architecture that can be explored using several visualization modalities and bioinformatics tools included in plusTipTracker. Graphical user interfaces enable novice Matlab users to track thousands of MTs in minutes. In this paper we describe the algorithms used by plusTipTracker and show how the package can be used to study regional differences in the relative proportion of MT subpopulations within a single cell. The strategy of grouping +TIP growth tracks for the analysis of MT dynamics has been introduced before (Matov et al., 2010). The numerical methods and analytical functionality incorporated in plusTipTracker substantially advance this previous work in terms of flexibility and robustness. To illustrate the enhanced performance of the new software we thus compare computer-assembled +TIP-marked trajectories to manually-traced MT trajectories from the same movie used in (Matov et al., 2010).

Keywords

microtubule; dynamics; tracking; live-cell; EB1; EB3

Introduction

Microtubules (MTs) are highly dynamic cytoskeletal polymers that stochastically switch between phases of growth, shrinkage, and pause, a behavior known as dynamic instability (Mitchison and Kirschner, 1984). While MTs are best known for their role in segregating chromosomes during cell division (Alberts et al., 2002), they also function in cell

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polarization and migration (Watanabe et al., 2005; Wittman and Waterman-Storer, 2001), intracellular trafficking (Caviston and Holzbaur, 2006), morphogenesis (Kirschner and Mitchison, 1986), and signaling to adhesions (Kaverina et al., 1998) and other organelles. In addition, MTs are a key drug target for the treatment of cancer (Giannakakou et al., 2000; Risinger et al., 2009) and other pathologies. Thus, quantitative characterization of MT dynamics in different cellular contexts is essential for our understanding of cell physiology and disease.

Researchers have measured MT dynamics in time-lapse images of fluorescently-labeled tubulin injected or expressed in living cells (Goodson and Wadsworth, 2004; Semenova and Rodionov, 2007). Because of the density of MTs in the cell body, this approach allows analysis of MT behavior only at the cell periphery. In recent years, imaging labeled +TIP proteins such as EB1 or EB3, which appear as comets streaking throughout the cell (Figure 1A), has replaced these experiments as a convenient technique for visualizing MT growth across all phases of the cell cycle (Gatlin et al., 2009; Perez et al., 1999; Piehl et al., 2004; Salaycik et al., 2005; Stepanova et al., 2003; Tirnauer et al., 2002a; Tirnauer et al., 2002b). +TIPs are a subset of MT-associated proteins (MAPs) that bind directly or indirectly at the tips of growing but not shrinking or paused MT plus ends, presumably by recognizing structural or chemical differences between the plus end and the lattice (Figure 1B) (Akhmanova and Hoogenraad, 2005; Dragestein et al., 2008; Galjart, 2010; Jiang and Akhmanova, 2010; Schuyler and Pellman, 2001). While early studies of +TIPs focused on qualitative observations, kymograph analysis and recently-developed automated particle tracking methods have enabled the estimation of nucleation rates (Piehl et al., 2004; Salaycik et al., 2005; Srayko et al., 2005) and the systematic measurement of growth speeds (Gatlin et al., 2009; Houghtaling et al., 2009; Kelly et al., 2010; Sironi et al., 2011; Smal et al., 2008).

The disadvantage of tracking MT dynamics by +TIP imaging is that comets reveal directly only the phases of MT growth. However, many of the MT-associated functions are determined by the dynamic switching between phases of growth, pause, and shrinkage. In principle, these transitions can be recovered from the growth trajectories: where two MT growth events are collinear and separated by a short time lag, it is likely that they belong to the same MT. By linking them together, parameters like shrinkage velocity or pause duration can be inferred (Figure 1C). Matov et al. showed proof of concept for this approach (Matov et al., 2010), but a globally optimal solution, integrated into a software package, is necessary for application of the method by the broader cytoskeleton community.

Here we describe plusTipTracker, a Matlab-based open source software package enabling +TIP comet detection, track reconstruction, track visualization, sub-cellular regional analysis, and MT subpopulation analysis. The package is available from <http://lccb.hms.harvard.edu> and runs on either Windows or Linux operating systems. Graphical user interfaces and several stand-alone support functions allow novice Matlab users to track and visualize thousands of MTs in minutes—a vast improvement for cytoskeleton biologists who have for many years painstakingly tracked MTs by hand. In addition, batch processing and bioinformatics tools make possible the development of cell-based screens from time-lapse images, enabling diverse applications from drug discovery to mechanistic cell biology.

Materials and Methods

Imaging and software

plusTipTracker has been tested on a wide range of +TIP live-cell data and performs optimally on image series filmed at 60x or 100x magnification with a frame rate ranging

from 0.5–2.0s. The software was developed and tested using Matlab R2008a. It can be assumed that the package runs on newer Matlab versions and the website <http://lccb.hms.harvard.edu> will feature regular updates.

Project setup, particle detection, tracking, and track post-processing are controlled by the plusTipGetTracks panel (Figure 2A), while visualization and some analysis tools are accessed via the plusTipSeeTracks panel (Figure 2B). Choice of optimal tracking parameter settings may be performed on a representative movie with the help of the plusTipParamSweepGUI tool (not shown). A full description of the software, along with a reference guide to all relevant functions, is available in (Applegate and Danuser, 2010), which is included with package download.

+TIP comet detection

Watershed segmentation—Fluorescently-labeled +TIPs appear as near-resolution-sized comets, hereafter referred to as particles, that vary in size, shape, and intensity over time and across regions of the cell. The image background signal also varies regionally and temporally, and due to the generally low signal-to-noise ratio (SNR), particles are often difficult to discern relative to the background. Because of particle heterogeneity and poor signal quality, application of a global detection threshold leads to an unsatisfactory number of false positives or false negatives. We avoid this problem by combining image enhancement with application of locally optimal thresholds.

First, raw +TIP images are enhanced using the Difference of Gaussians (DoG) approach (Spring et al. 2006). To create DoG images, each raw image (Figure 3A) is filtered twice: first with a small Gaussian kernel (default value $\sigma_1 = 1$ pixel, Figure 2A blow-up) to eliminate high-frequency intensity fluctuations due to noise, and second with a larger Gaussian kernel (default value $\sigma_2 = 4$ pixels, Figure 2A blow-up) to eliminate larger-scale variations in cell background. Subtraction of the second Gauss-filtered image from the first results in a bandpass-filtered image with a relatively uniform background and suppressed noise, but particles can vary significantly in size, shape, and intensity, and their images may overlap. To extract the coordinates of these particles, we use a watershed-based method: the image is treated as a three-dimensional intensity landscape (Figure 3B) to which gradually lower thresholds are applied sequentially. Each detected particle is allowed to grow in area as the threshold drops from the maximum gray level value until it either begins to merge with another particle, at which point the two particles are retained separately, or it reaches the minimum threshold value (Figure 3C).

The minimum threshold, m , must be high enough that fluctuations in the background are not accepted as particles, but low enough that faint particles are not discarded. Similarly, the threshold step size, s , between threshold levels must be small enough to capture closely-apposed particles but large enough that two local maxima within an individual comet are not detected as separate particles. The latter situation often occurs at too-high expression levels where +TIPs accumulate in extended comets or with certain +TIPs that have a high affinity for MTs. To estimate these two parameters, the image intensity standard deviation is first calculated for each DoG image in the time series. Then, for each image i , the standard deviations from frames $i-2$ to $i+2$ are averaged to yield its threshold step size s . The minimum threshold m for each image is defined as $K*s$, with $K = 3$ as a default setting to achieve detection with an expected 1% false negatives (Figure 2A blow-up).

The algorithm requires no assumptions about particle size, shape, or intensity. Thus, in contrast to our previously published work on +TIP tracking (Matov et al., 2010), for routine analysis the algorithm is free of control parameters. Robustness of the detection with default parameter values has been qualitatively confirmed by visual inspection across a large

number of movies of varying image quality (not shown). Nevertheless, the software does present the user with the possibility to change the DoG filter settings σ_1 and σ_2 and the threshold multiplication factor K . Values greater than 1 for σ_1 and less than 3 for K may improve the detection of comets with low SNR. Values greater than 3 for K may reduce the number of false positive particle detections when the cytosolic background signal contains some structure. Such false positives may also be oppressed by increasing the value of σ_2 . To some extent an increase beyond its default value of 4 may enhance the detection performance when the comets are significantly elongated. This occurs either with +TIPs that have a high affinity for MTs or with high overexpression of the fluorescently-labeled +TIP, both resulting in an increased decoration of the MT lattice.

Anisotropic Gaussian comet model fitting—For comets with highly elongated tails, the application of local optimal thresholds as described above has the tendency to generate false positives (Figures 3E,F; 4B,C). To resolve this issue, we implemented an alternative detection algorithm which relies on the implicit model of a comet image as a two-dimensional anisotropic Gaussian. In the first step, the algorithm identifies candidate comets by applying steerable filters (Jacob and Unser, 2004) and subsequently detecting local maxima in the filter response. The order of the filter is 2 in order to detect comets as ridges. The filter depends on a control parameter defining the scale of filter support. We set this parameter to 2 times the standard deviation of the Gaussian approximation of the microscope point spread function (PSF; Thomann et al., 2002), as the width of the comet is defined as the diffraction limit of the microscope whereas the length exceeds the dimensions of the PSF. In the second step, each local maximum is fitted by a two-dimensional anisotropic Gaussian. Besides the position of the putative comet, the fit procedure also returns the eccentricity of the comet and the amplitude of the signal, including estimates of the uncertainty of both of these free parameters. The detection method then uses a statistical significance test to identify comet models with significant amplitudes as bona fide +TIP comets. The software interface allows the user to tune the α -value (i.e. 1 – the confidence probability) required for the acceptance of a comet (Figure 4A).

Independently of the choice of particle detection method, the software generates a file in the 'feat' (i.e. feature) subfolder called 'movieInfo.mat'. The file contains a list of particle positions, intensities, and areas for each frame in the time series. The user of plusTipTracker can, of course, write another detection module that could then interface with the tracking modules via this file.

+TIP comet tracking

In this paper, a trajectory is defined as the real path of the MT plus end over time, while a compound track is defined as the reconstruction of that trajectory by joining directly-observable growth phases, referred to as growth sub-tracks, from the same MT based on spatial and temporal cues. Thus a compound track is composed of n growth sub-tracks and $n - 1$ inferred sub-tracks, corresponding mainly to phases of pause or shrinkage. The inferred sub-tracks can also represent a continuation of growth in the event of a detection false negative or a comet moving temporarily out of the focal plane. Whether an inferred sub-track represents pause, shrinkage, or growth depends on the relative orientation and speed of its flanking growth sub-tracks (see post-processing section in Materials and Methods).

Trajectory reconstruction is accomplished using the single particle tracking (SPT) framework described in (Jaqaman et al., 2008), which presents a generic tracking solution for applications where high particle density, particle motion heterogeneity, and temporary particle disappearance present challenges. Briefly, tracking occurs in two steps. In the first step, corresponding particles in consecutive frames are linked to create growth sub-tracks. In

the second step, growth sub-tracks matching certain spatial and temporal criteria are linked, thus defining compound tracks. (These two steps are analogous to the “tracking” and “clustering” steps in (Matov et al., 2010).) The first step, particle linking, is temporally greedy but spatially globally optimal, i.e. the best possible set of links between particles is picked, taking into account the entire population of particles in two consecutive frames. The second step, sub-track linking, is both temporally and spatially globally optimal, i.e. the best possible assignment of sub-tracks into compound tracks is picked, taking into account the entire population of growth sub-tracks throughout the movie.

Both optimization steps are achieved by solving a linear assignment problem (LAP) (Burkard and Cela, 1999; Jonker and Volgenant, 1987). The solution to an LAP identifies the set of links yielding the lowest overall cost, given a list of potential particle or sub-track pairs and a list of their associated costs. Importantly, the LAP solution deals with competing pairs by determining which set of links is *overall* best; this does not necessarily lead to the lowest-cost link for each individual particle or sub-track, but includes compromises with neighboring links as needed.

We have defined cost functions for both the particle and sub-track linking steps by defining models of MT dynamics. For the particle linking step, we modified the linear motion cost function described in (Jaqaman et al., 2008) as follows: 1) we removed the possibility of back-and-forth linearly diffusive motion, accounting for the unidirectional growth of MTs, and 2) we changed the initialization function for the motion-propagating Kalman filter to allow a large search radius for particles upon their first appearance. The latter modification was necessary since comet displacement between frames may be much larger than would be captured by the more conservative search radius required once track directionality has been established.

While most sub-tracks created during particle linking are unidirectional (Figure 5A), some mistakes are inevitable because of the circular search regions and the temporally-greedy particle linking approach. It is empirically known that MTs rarely, if ever, abruptly change direction during growth; thus sub-tracks that deviate from linearity are split in two. Any resulting sub-tracks which are too short after splitting (less than three frames) are discarded, while the rest become candidates for sub-track linking.

The purpose of the sub-track linking step is to join +TIP-marked growth phases that have a high likelihood of belonging to the same MT. From observations of fully-labeled MTs in live-cell images, it is clear that consecutive growth phases for a single MT are approximately collinear and oriented in the same direction. Thus only growth sub-tracks matching this criterion are considered for linking. In addition, the time lag between the end of the first sub-track and the beginning of the second must also be reasonably short to avoid linking growth phases from distinct MTs which happen to grow along the same path, a phenomenon frequently observed in MT bundles associated with filopodia and with adhesion structures along the cell edge.

Gaps between growth sub-tracks occur in either the forward or backward direction (relative to the first sub-track in the sequence; see Figure 5B) depending on the underlying MT dynamics and detection performance. Gaps occur in the forward direction as a result of 1) temporary comet disappearance from the focal plane, 2) a false negative during detection (common when MT plus ends cross one another), or 3) a MT pause. Gaps occur in the backward direction as a result of 1) thermal fluctuation of the plus end position during pause, or 2) MT catastrophe (transition from growth to shrinkage) followed by rescue (transition from shrinkage to growth). Interpolation of MT plus end position during forward

and backward gaps (henceforth referred to as “fgaps” and “bgaps”, respectively) generate the inferred sub-tracks between growth sub-tracks.

In order to close gaps robustly, we must 1) carefully determine how candidate sub-tracks for linking should be chosen, and 2) define a cost function that results in an unbiased selection of forward and backward gaps. Candidate selection is driven by MT track geometry. For each sub-track i , a search region is defined by the union of a small circle and two cones (Figure 5B). The circle is centered on the sub-track’s last detected position, P_{i_end} , and has a radius, r_{fluct} , related to the detection uncertainty and positional fluctuation of the plus end that may occur during MT pause (typically 1–2 pixels). A wide cone (± 25 – 45° angle) extends forward, parallel to the average velocity vector from the sub-track’s final three time points, while a narrow cone (± 10 – 15° angle) extends backward, following along the sometimes curved sub-track. The radius of each cone is proportional to the expected displacement of the MT during the gap, given the known distribution of growth speeds from the particle linking step and a user-set shrinkage speed-to-growth speed ratio (“maximum shrinkage factor”), which exists because MT shrinkage speeds are usually faster than growth speeds (Komarova et al., 2009; Matov et al., 2010; Shelden and Wadsworth, 1993). Any sub-track j originating in the search region and beginning within Δt_{max} , the maximum-allowable gap duration from P_{i_end} , is a candidate for linking to sub-track i . Candidates originating in the circle or forward cone will generate fgaps (Figure 5B, green sub-track), while candidates originating in the backward cone will generate bgaps (Figure 5B, blue sub-track). In addition, the initial direction of growth of a candidate j must not deviate significantly from sub-track i ’s direction at P_n , the point nearest the candidate track’s initiation point, P_{j_start} . This criterion is based on the observation that the lattice of a MT stays approximately stationary while the plus end grows and shortens.

The cost of linking sub-track i with any candidate j , whether in the forward or backward direction, is calculated from four parameters: $d_{||}$, $d_{\perp}$, θ , and Δt_{gap} (Figure 5B). The first, $d_{||}$, denotes the distance along sub-track i from P_{i_end} to P_n . For forward gaps, this is the parallel component of the displacement vector between P_{i_end} and P_{j_start} ; for backward gaps, this is the length back along the actual path traced out by the MT in previous time points from P_{i_end} to P_n (extrapolating beyond P_{i_start} if the sub-track is too short, as in the case of the blue track in Figure 5B). The second parameter, $d_{\perp}$, denotes the shortest distance between P_{j_start} and sub-track i . For forward gaps, this is the perpendicular component of the displacement vector between P_{i_end} and P_{j_start} ; for backward gaps, this is the distance between P_n and P_{j_start} . The third parameter, θ , is the angle between sub-track i ’s direction at P_n and candidate j ’s direction at P_{j_start} . The value for θ is the same whether sub-track j is in the forward or backward cone. The fourth parameter, Δt_{gap} , is the duration of the gap expressed in number of frames between P_{i_end} and P_{j_start} . The cost C of linking sub-track i and candidate j is defined as

$$C_{ij} = 1.1^{\Delta t_{gap}} \times (d_{||}^* + d_{\perp}^* + [1 - \cos(\theta)]),$$

where 1.1 is an empirically chosen value and $d_{||}^*$ and $d_{\perp}^*$ represent normalized values of $d_{||}$ and $d_{\perp}$. Normalization ensures equal weights for the distance- and orientation-based components in the cost function. It is accomplished by dividing $d_{||}$ and $d_{\perp}$ of the specific candidate link between sub-tracks i and j by the 99th percentile of the distribution of all $d_{||}$ and $d_{\perp}$ pooled from all potential sub-track pairs. Thus, links with longer time gaps, larger displacements, and larger angular deviations are penalized by a higher cost. The overall best set of links is chosen by minimizing the global cost via solution of the LAP.

The validity of the sub-track linking cost function can be verified by visual inspection of track overlays (using plusTipSeeTracks; Figure 2B, 'Track Overlays' panel) and movies ('Track Movies' panel), and by plotting the distributions of forward and backward costs (Figure 5C). The cost distribution for all potential links (Figure 5C, left) decreases smoothly, suggesting that the four parameters used to calculate the cost are normalized appropriately. In addition, the cost distribution is not dominated by either forward (green) and backward (blue) costs, suggesting that neither type of link is intrinsically favored by the algorithm during candidate selection. This relative distribution is preserved for sub-tracks with only one potential link (Figure 5C, right), verifying that links which are being made with and without competition have the same characteristics. This indicates a high consistency among the link configurations in cellular areas of variable MT density.

The output of tracking is a file in the 'track' subfolder called 'trackResults.mat,' which contains a list of compound tracks. Each track is composed of one or more sequential growth sub-tracks, which in turn are composed of particle index lists corresponding to the results obtained during detection.

Control parameters for tracking

plusTipTracker requires several control parameters for tracking (Figure 2A, Tracking Settings box; Supplementary Table 1) that tune the size of the search regions and time window for candidate selection. Here we describe how these tracking parameters affect candidate selection, while in (Applegate and Danuser, 2010) we provide practical guidance on how to choose the appropriate settings based on image acquisition and biological considerations.

- *Maximum gap length.* This parameter corresponds to Δt_{max} , the maximum-allowable gap duration in frames between P_{i_end} and P_{j_start} . This value should be long enough to capture a significant portion of pause and shrinkage events but short enough to avoid both incorrect links and a combinatorial explosion in the sub-track linking step due to the consideration of too many candidates.
- *Minimum sub-track length.* This parameter sets the minimum number of consecutive frames over which a comet must be tracked for it to count as a sub-track. This value is typically set at 3 to filter out sub-tracks of one or two frames that may arise from detection false positives.
- *Search radius range.* This range defines the lower and upper bounds on the search radius estimated by the Kalman filter for each particle from its motion history (Jaqaman et al., 2008). In brief, the Kalman filter predicts where each particle will appear in the next frame given its behavior in previous frames. The tracking algorithm then searches for particle linking candidates within a search radius around the predicted position. The size of the search radius for each particle is automatically estimated based on the frame-to-frame variation in each particle's motion characteristics: it becomes smaller if a particle moves at a constant speed over several frames, reflecting a high degree of certainty in the predicted position, while it becomes larger if the particle alternates between fast and slow motion, reflecting a lower degree of certainty.
- *Maximum forward and backward angles.* These parameters control the acuteness of the forward and backward cones.
- *Fluctuation radius.* This parameter, corresponding to r_{fluct} , controls the size of the circular portion of the search region, defining how much a pausing MT can fluctuate sideways during a pause event.

- *Maximum shrinkage factor.* This parameter is the multiplier to the maximum growth speed which controls the upper bound for plus end displacement during shrinkage; i.e., the maximum radius of the backward search cone for sub-track i is the product of v_{max} (the 99th percentile of all growth speeds found during particle linking), the time gap Δt_{gap} , and the max shrinkage factor.

Post-processing of tracks

To extract information about MT dynamics, the particle and sub-track linkages generated during tracking must be parsed and analyzed. For compound tracks containing two or more growth sub-tracks, the gaps between them are coded as forward gaps (fgaps) or backward gaps (bgaps), depending on their relative spatial orientation. MT plus end coordinates are then interpolated during gaps assuming constant velocity, and the instantaneous velocities for each compound track are determined by calculating the displacement between consecutive frames.

Because gaps can arise for reasons other than true pause or shrinkage, we stratify the gap speed distributions. MT plus end displacements during an fgap that yield a speed greater than 70% of the growth speed just prior to comet disappearance are strong indicators of fgaps representing continuation of growth. Accordingly, we reclassify such fgaps as growth phases. These fgaps usually arise due to detection failures or particles moving temporarily out of the focal plane. The remaining fgaps are assumed to be true pause events. Nonzero speeds are expected in this population due to the short lag between MT rescue and comet reappearance commonly observed in reconstituted systems (Bieling et al., 2007) and in live cells (Dragestein et al., 2008). Bgaps are similarly analyzed to determine which ones most likely correspond to true shrinkage. If a MT's plus end displacement during a bgap yields a speed slower than the 95th percentile of the speed distribution of fgaps classified as bona fide pause events, we assume with a confidence of 95% that the bgap represents a pause rather than shrinkage. Such cases are often due to positional jitter or polymer drift during a pause, which can cause the comet to reappear in the backward cone of the search region. The remaining bgaps are classified as shrinkage events.

After reclassification of the inferred sub-tracks, track profiles are represented in a matrix (similar to Figure 5D), where the columns represent the following: compound track index, track type (e.g. growth, fgap), starting frame, ending frame, average speed ($\mu\text{m}\cdot\text{min}^{-1}$), lifetime (frames), and total displacement (pixels). While each fgap reclassified as continuation of growth is maintained as a distinct sub-track in this profile matrix, for statistical analyses of MT growth it is merged with its flanking growth sub-tracks. Similarly, each bgap classified as a pause is maintained as a distinct sub-track in the profile matrix but is grouped with the fgap pause events in subsequent statistics.

Supplementary Table 2 lists the statistics calculated during post-processing related to MT dynamics and tracking performance. These statistics are stored in a Matlab structure called 'projData.mat,' which is the most important output of plusTipTracker. All visualization and higher-level analysis functions of the plusTipTracker package make use of the projData structure. This structure is also the place for more advanced users to interface their own post-processing functions with the package.

Bioinformatics and visualization functions

plusTipTracker includes several support functions to facilitate the comparison of data between individual projects or between experimental groups. Data for each project may be exported for analysis in Excel or other programs, or the user may create groups of projects to

be analyzed together using several different support functions (see function guide in (Applegate and Danuser, 2010)).

The plusTipSeeTracks interface offers a number of ways to visualize the data, including interactive track overlays (e.g. Figure 5D), movies of individual tracks or regions-of-interest (ROIs) (e.g. Supplementary Movies 4–7), and movies where comets are color-coded by speed and phase (e.g. Supplementary Movie 8, growth phases appear as circles, fgaps appear as triangles, and bgaps appear as squares). For some applications, it is desirable to determine how MT growth varies from one region of the cell to another. This may be accomplished using the sub-ROI selection tool (Figure 2B, ‘Sub-ROIs), which enables the user to manually or automatically divide the cell into multiple regions and compare tracks between them. Sub-ROI analysis elucidates how MTs behave on average in a given region of the cell, but it is also useful to analyze the spatial distribution of classes of MTs with specific properties. This kind of analysis can be performed with the quadrant scatter plot tool (Figure 2B, ‘Quadrant Scatter Plots’). Sub-tracks are divided into four subpopulations based on two MT dynamics parameters and a “split value” for each. Extensive descriptions of these tools are included in (Applegate and Danuser, 2010). A final kind of track visualization can be performed to analyze spatial distributions of MT dynamics. Using the plusTipPlotResults function, speed, lifetime, and displacement maps are generated for each of the growth, fgap, and bgap sub-track populations. These reveal at a glance how the three parameters vary for each track type across the cell. The initiation and termination sites for fgaps and bgaps are also plotted to reveal spatial patterns of MT catastrophe and rescue.

Simulations of synthetic +TIP movies

To benchmark plusTipTracker, we generated synthetic movies of +TIP comets and compared the detection and tracking results to the simulation ground truth. We varied the comet shape, from symmetric to highly-eccentric, the MT density, and the SNR.

The MT network was simulated as follows: The MT organizing center (MTOC) was placed in one corner of the image and the cell area appearing in the image was taken as a quarter-circle with center at the MTOC and radius = image edge length L . MTs emanated radially from the MT organizing center, with randomly assigned initial lengths between 0 and L and direction angles between 0 and $\pi/2$. The MTs underwent dynamic instability within the cell area, simulated using a Gillespie-type kinetic Monte Carlo algorithm (Gillespie, 1977). The MT growth and shrinkage speeds and times followed gamma-distributions (Odde et al., 1995), with means and standard deviations reflecting experimentally observed values: growth speed = 20 ± 2 $\mu\text{m}/\text{min}$, shrinkage speed = 30 ± 3 $\mu\text{m}/\text{min}$, growth time = 20 ± 4 s, and shrinkage time = 10 ± 4 s.

To benchmark the detection algorithms, +TIP comet movies were generated from the dynamic MT network with the following parameters: image size 512x512 pixels, pixel size 0.1 μm , MT density 10^{-3} MTs/pixel (or 0.1 MT/ μm^2), frame rate 1 Hz. This MT density was similar to the densities observed experimentally. Comet images were approximated by 2D Gaussians with a short axis standard deviation of 1.5 pixels matching the PSF of the microscope and a long axis generating an eccentricity between 1 (i.e. circular) and 5. Comets were placed at the plus-tips of growing MTs with the long axis parallel to the direction of MT growth. Shrinking MTs had no +TIP comets. Gaussian noise was then added to the movie frames to simulate SNR in the range 2 to 10.

To benchmark the tracking algorithm, we varied the density of MTs between 10^{-5} and 10^{-2} MTs/pixel (or 10^{-3} to 1 MT/ μm^2). The movies were generated with a sampling rate of 1 Hz, with total movie length = 100 frames. To benchmark the tracking algorithm independently of the detection algorithm, we directly derived from the simulated +TIP

trajectories a list of particle positions per frame. Noise effects were mimicked by deleting a fraction of particles from the list, using the relationship between SNR and false negatives identified by the benchmarking of the detection algorithms. We did not include false detection positives in the simulations as those were rare with the anisotropic Gaussian detection algorithm and could be mostly eliminated by the tracker which only retained tracks that lasted for at least three frames.

Results and Discussion

The software has been applied to a wide range of data from diverse cell types and +TIP markers, and imaged with different acquisition settings (although all in 2D). Of note, the software has been applied to cells in 3D environments where light scattering produces relatively high background signals and thus reduces the achievable SNR (Myers et al., 2011). Moreover, the software is also being employed in high-content screens, where large movie data sets are processed without user intervention (unpublished).

In the following sections we illustrate key features of the software functionality based on a movie of a human endothelial cell expressing EB3-EGFP. The movie contains a single polarized cell (Supplementary Movie 1) with large, bright, long-tailed comets in the cell body and small, faint, rounded comets near the periphery. The cell background is also brighter in the cell interior than at the periphery, where the cell edge is not distinguishable by eye. Thus particle heterogeneity in size, intensity, and shape, as well as large-scale image intensity variation, present challenges for automated detection. The real space pixel size is $0.105\mu\text{m}$ and the frame rate is 2.0s. Images have been cropped to a size of 878×957 pixels, and 75 frames are used for the analysis.

Fast, robust, and adaptive detection of +TIP comets

We first investigated the performance of plusTipTracker's comet detection by watershed segmentation. This algorithm processed the movie at a rate of $\sim 5\text{s}/\text{frame}$ on a 64-bit, 3 GHz Xeon processor with 16 GB RAM and it detected an average of 394 comets per frame. Robustness was assessed qualitatively by visual inspection (Supplementary Movie 1) and quantitatively by comparing results to manual detection (Figure 3D). The percentage of false positives, $100(1-M/D)$, and the percentage of false negatives, $100(1-M/G)$, where D is the number of computer-detected comets, G is the number of manually-detected comets, and M is the number of matches, were both below 4%. False positives (yellow circles) most often appeared as secondary peaks on comets with a bright, extended tail, while false negatives (cyan circles) occurred when particles were out of focus, extremely small, or too closely apposed to each other to be resolved as distinct (white arrow). This low error rate was achieved without any user intervention or adjustment of control parameters. Over the course of the movie, image intensity decreased dramatically due to photo-bleaching; the average intensity in the last frame was 28% lower than in the first (Supplementary Movie 1). Yet, the large number of particles detected per frame remained fairly constant (394 ± 14.0 particles), indicating robustness of the detection against global signal variation.

We also determined the error rates of watershed segmentation using simulated movies (Figure 3E,F; Supplementary Movie 2). As on real-world data we found that for comets with eccentricity < 2 both the false positive and the false negative rates were 5% or below. Remarkably, for SNR values greater than 3 the error rates were almost independent of the noise level. However, for images with an SNR in the range 2 – 6 the performance deteriorated rapidly when the eccentricity increased. With such comets the DoG filter response, which implicitly relies on a particle model with symmetric intensity distribution, often generated two or three significant maxima along a single comet. Therefore, the false positive rate rapidly increased with increasing eccentricity (Figure 3E).

To circumvent these limitations plusTipTracker offers an alternative detection method, which relies on an explicit model of the comet as an elongated image feature (Figure 4). Elongated comets occur either with +TIPs that have a high affinity for MTs or with substantial overexpression of the fluorescently-labeled +TIP. Both scenarios yield an increased decoration of the MT lattice by the +TIP. Figures 4B,C compare in a zoomed view the watershed-based segmentation and the segmentation by anisotropic Gaussian model fits on a movie with higher expression of the +TIP EB3. A comparison of the performance on the full movie is presented in Supplementary Movie 3. Clearly, under these conditions a method that modeled the eccentricity of the comets delivered much better results. However, it should be noted that the explicit model fit used for this segmentation was computationally more costly. Also, it tended to produce more false negatives than the watershed-based segmentation in areas of high comet density. Finally, the localization of an elongated feature was less precise than for a feature with isotropic intensity. For all these reasons, as well as for reducing the risk of actual biological perturbation of the cell system, it is advisable that the expression levels of +TIPs are kept low to allow the use of watershed segmentation. As mentioned before, this approach is supported by the robustness of the segmentation against low SNR, albeit primarily for isotropic comets.

One cell, thousands of tracks

The example movie chosen presents a significant challenge for any particle tracking algorithm due to the high density of fast-moving particles. A critical parameter used to define the difficulty of a tracking problem is the ratio between average frame-to-frame particle displacement, d_{FF} , and average nearest-neighbor distance, d_{NN} . Most algorithms produce a high number of false positives when the ratio exceeds 0.25 and fail completely at a ratio of 0.5 (Jaqaman and Danuser, 2009). Our specific test data set had a d_{FF} to d_{NN} ratio of 0.56, highlighting the combined power of motion propagation and global optimization performed by plusTipTracker (Jaqaman et al., 2008). In addition, sub-track linking based on MT motion models allowed the conservative inference of hundreds of pause and shrinkage events, giving insight into the regulation scheme of MT dynamics across the whole cell.

Tracking and post-processing for the example movie took 85 and 10 seconds, respectively, on the above specified processor configuration. Clearly, this is many orders of magnitude faster than manual tracking and provides more comprehensive coverage of the entire cell than some semi-automated methods (Piehl et al., 2004; Salaycik et al., 2005).

Here we discuss a few of the many statistics related to MT dynamics which were saved in the projData Matlab structure (see Supplementary Table 2). Over 75 frames (150 s), 2,540 growth sub-tracks were linked into 1,479 compound tracks, yielding 911 fgaps and 305 bgaps. The mean growth speed was $21.7 \mu\text{m}\cdot\text{min}^{-1}$, while the mean bgap speed was $26.3 \mu\text{m}\cdot\text{min}^{-1}$. These values are fast compared to many examples in the literature, but their ratio is consistent as it has long been observed that shrinkage rates *in vivo* are higher than growth rates (Shelden and Wadsworth, 1993; Waterman-Storer and Salmon, 1997). Growth rates are known to vary significantly depending on cell type, serum conditions, and the substrate (Dhamodharan and Wadsworth, 1995). We chose this particular data set in part because of the unusually fast MT growth, imaged at a slow sampling rate (0.5 Hz). This represents one of the most difficult tracking configurations we have so far encountered in our applications of the software. We conjecture that most tracking problems in MT cell biology will be easier.

Average fgap lifetimes (13.s) were slightly longer than growth phase lifetimes (12.9s) and over a second longer than bgap (shrinkage) lifetimes (12.0 s). These values are on par with corresponding durations measured for other cell types (Dhamodharan and Wadsworth, 1995; Salaycik et al., 2005), though the results are not directly comparable for several reasons (see

Algorithm Validation and Conclusions sections). The average displacements for growth, fgap, and bgap sub-tracks were 5.3 μm , 1.7 μm , and 5.1 μm , respectively (Figure 6A, top row). The growth lifetime distribution was exponential, as expected for a stochastic process like MT dynamic instability. The fgap and bgap lifetime distributions were restricted, by definition, to a maximum length of 12 frames (the maximum gap length parameter set for tracking), or 24 s (Figure 6A, top row). In contrast, some growth sub-tracks were extremely long lived (2% > 46 s). For illustration, Supplementary Movies 4–7 show representative trajectories of comet dynamics. Supplementary Movie 6 contains a comet growing continuously for 78s.

The frequencies of fgap and bgap, whether measured in time or length, were similar for this movie (0.07 s^{-1} and 0.17 μm^{-1} for fgap; 0.08 s^{-1} and 0.18 μm^{-1} for bgap). The frequencies in time (or length) were calculated as the number of fgaps or bgaps divided by the sum of all growth lifetimes (or displacements) prior to the fgaps or bgaps. Hence, fgap (or bgap) frequency is the inverse of the average lifetime or displacement a MT spends growing before entering an fgap (or bgap). To test whether fgaps or bgaps followed a particular subpopulation of growth sub-tracks, e.g. they would preferentially follow long phases of growth or fast growth, we performed the following analysis: From the full population of growth sub-tracks in the movie, we randomly picked 911 samples, i.e. the size of the random sample population matched the population of 911 growth sub-tracks that was actually followed by an fgap. We then calculated the lifetime and net displacement of the random growth sub-track samples and compared their distribution to the lifetimes and displacements of the population of sub-tracks followed by fgaps. We repeated this procedure 1000 times and could not find a statistically significant difference between the distributions. The same applied to the analysis of growth sub-tracks followed by bgaps. This suggested that MT growth duration and distance prior to gaps were randomly distributed. Importantly, this does not mean that these events are unregulated. On the contrary, maps of the location of the initiation and termination sites for fgaps and bgaps reveal a clear preference for fgaps and bgaps at the cell periphery (Figure 6B), suggesting the presence of spatial cues in the regulation of MT dynamics.

On average, MT plus ends moved 1.7 μm during fgaps (pause) lasting 13.1 s. Displacement of plus ends during pause is expected due to the latency between MT rescue events and comet reappearance (Figure 1C and (Matov et al., 2010)).

Reports in the literature vary on the percent of time MTs spend growing, pausing, and shrinking. Typically, MTs spend most of their time growing, the least of their time shrinking, and some intermediate time in a paused state (Wadsworth, 1999; Waterman-Storer and Salmon, 1997). The MTs in our example movie followed this trend, spending 67.7% of their collective lifetime in growth, 24.8% in pause (fgap), and 7.5% in shrinkage (bgap). At an individual level, compound tracks containing a gap spent, on average, 35.4% and 22.8% of their lifetimes in pause and shrinkage, respectively. The individual track percentages were higher than the collective percentages since 52.1% of tracks in the collective group contained no fgaps or bgaps. A large portion of these isolated growth tracks transitioned to terminal shrinkage events. Due to the nature of the tracking method, terminal shrinkage (or pausing) events could not be detected. Therefore, the aforementioned breakdown of MTs in a growing, pausing, and shrinking sub-population was biased towards a larger growing population.

Sub-cellular organization of MT growth speed

The data in Figure 6A (Rows 2–4) show a clear pattern of spatial regulation for MT growth speed: MTs grow faster in the cell center than at the periphery—a feature of MT regulation that has been impossible to quantify with tubulin labeling. We suspect that this is a critical

cue in cytoskeleton regulation, related to the control of cell polarity and organization of organelles. To investigate the spatial regulation of MT dynamics further, we complemented plusTipTracker with a 'Sub-ROIs' tool (Figure 2B). The tool supports either manual definition of cellular regions or the automatic division of the cell area into a center, front, back, left, and right region (Figure 7A). At the present time, we extract only growth sub-tracks during sub-region analysis.

The growth speed distributions for each sub-cellular region (Figure 7B) suggest that there are indeed distinct modes of spatial regulation across the cell. The mean growth speeds match the pattern observed visually in Figure 6A: MTs in the center are fastest, while those at the periphery are slower. However, it can also be seen that MTs in the front of the cell grow on average faster than in the rear or sides. Insight into regulation may also be gained from the shapes of the distributions. Unlike the cell rear and side distributions which are unimodal, the distributions at the cell front and center are bimodal, indicating the existence of either two distinct classes of MTs or two distinct MT regulatory regimes. Interestingly, in the center region the separation of fast- and slow-growing MTs is more pronounced than in the front region. We have used the plusTipTestDistrib function to statistically compare the pairs of distributions and their means (Figure 7C). Below the diagonal of the discrimination matrix are the results of a mean-subtracted Kolmogorov-Smirnov (KS)-test, indicating the percent confidence that the distributions are different. The test statistic was generated by bootstrapping, calibrating the p-values by sub-sampling one distribution against itself, in order to account for the hyper-sensitivity of the KS-test with large sample populations. Above the diagonal are p-values for a permutation t-test of the means. As expected from the histograms, the center distribution is significantly different from the other four, while the back is most similar to the two sides. Combined with molecular perturbation, this kind of sub-cellular region analysis will be extremely useful for investigating the mechanisms of spatial regulation of the MT cytoskeleton.

Relative distributions of different MT classes

It is well established that MT switching frequency between growth, shrinkage, and pause varies across the cell: most MTs nucleated from the centrosome grow persistently until reaching the cell periphery, where they begin to oscillate between growth and shrinkage (Komarova et al., 2002). In contrast, certain "pioneer" MTs enter the lamellipodium and continue to grow persistently, often parallel to the cell membrane (Waterman-Storer and Salmon, 1997). Catastrophe frequency is asymmetric in polarized, motile cells, with more catastrophes occurring at non-leading edges (Wadsworth, 1999) and at the back of the cell (Salaycik et al., 2005) compared to the leading edge. Some post-translationally modified MTs are extremely stable and form an array oriented in the direction of migration (Gundersen and Bulinski, 1988), while MTs that target adhesion sites can be stabilized (Kaverina et al., 1998) or undergo high frequencies of paxilin-dependent catastrophe (Efimov et al., 2008; Wittmann et al., 2003). In addition, MT persistence is differentially regulated in different parts of the cell by Rho family GTPases (Wittman and Waterman-Storer, 2001), and +TIP proteins themselves, while often being used as a marker for growing plus ends, also regulate MT dynamics (Komarova et al., 2009).

To investigate the spatial organization of dynamic switching behavior, we implemented the 'Quadrant Scatter Plots' tool (Figure 2B), which allows the classification of sub-tracks into four subpopulations on the basis of two dynamics parameters. For the purposes of illustration, we chose growth speed and growth lifetime, and used the mean values for each ($21.7\mu\text{m}\cdot\text{min}^{-1}$ and 12.9s) to split the data (Figure 8A). The four subpopulations of the scatter plot are displayed as overlays on the cell image, either combined (Figure 8B) or separately (Figure 8, C-F). These plots clearly indicate different localizations for MTs with different dynamic properties. Further, by extracting the tracks in each previously-selected

sub-ROI (Figure 8G), the relative proportions of the four subpopulations in each sub-ROIs can be compared to those of the whole cell (Figure 8H).

As expected, the central region is dominated by fast-moving MTs (43% yellow and 31% blue), but interestingly almost 70% of sub-tracks in this region are not particularly long-lived (25% red and 43% yellow), conflicting with the traditional view that MTs persist from the centrosome to the cell edge before becoming highly dynamic. The abundance of short-lived tracks in the cell body has not been previously reported as studies relying on manual tracking are biased towards long trajectories that are easy to follow by eye. These data illustrate the importance of complete measurements of MT behavior in order to dissect the complex and spatially heterogeneous regulation of the MT cytoskeleton.

MTs at the back of the cell have the highest switching rates (75% red and yellow), whereas MTs at the front are the most persistent (only 62% red and yellow). Again, these data contradict the common notion of particularly dynamic MTs at the cell front. We suspect that this difference originates in part from the fact that at the cell front MTs are less dense and thus more completely tracked by hand whereas in other areas of the cell the higher dynamics has been missed due to a selective measurement of the more stable MT sub-population.

Algorithm validation with synthetic movies

Following the extensive validation of the underlying particle tracking software published in (Jaqaman et al., 2008), we repeated some of these performance tests on synthetic +TIP comet movies, which simulate the peculiar conditions for sub-track linking that occur with MT pausing and shrinking. The dynamics of the MTs were defined by the average behavior of MTs in our experimental data (see Methods). We then varied the MT density, resulting in conditions with average d_{FF} to d_{NN} ratios between 0.03 and 0.71. We also varied the percentage of false negative detections between 0 and 20%. Note that this is much higher than the false negative rates of the detector, as shown in Figure 3E,F. Missing detections can also occur with temporary out-of-focus movements of comets and thus the effective challenge for the tracker is higher than what is induced by limitations of the detector module. Figure 5E indicates that the frame-to-frame linking was essentially error-less in these ranges of the simulation parameters. The forward gap detection produced very low false positive rates, but reached rates of a few percent when 10% of the comets were missing. Interestingly, both performances were nearly independent of the MT density, again highlighting the combined power of motion propagation and global optimization performed during the gap closing (Jaqaman et al., 2008). As expected, the detection of backward gaps introduced by shrinkage events was the least accurate. False positives and false negative rates reached ~20% when 10% of the comets were missing. False positives originated in this case from growing MTs that followed the same axis behind a MT that had switched into a phase of shrinkage. False negatives originated in very long shrinkage phases that could no longer be interpolated. Of note, this error rate can be reduced by increasing the maximum gap length. However, this will have to be traded against an increased rate of false positives, both in forward and backward gaps. In summary, these simulations indicate that the algorithm is very stable with respect to variations in MT density; yet, it is essential to minimize detection misses, both by generating high SNR movies and by choosing samples where the out-of-focus motion of MT ends is limited.

Algorithm validation with dual-wavelength movies of +TIP markers and tubulin

We also assessed the performance of plusTipTracker, and especially the one in linking sub-tracks in time-lapse image series recorded of cells expressing both EB1-EGFP and mCherry-tubulin (Figure 9A). The same image sequence was used to evaluate the proof-of-concept approach for track clustering described in (Matov et al., 2010). Frames were sampled at

intervals of 0.6 s and the effective pixel size in the specimen space was 107 nm. Tracking parameters were chosen using plusTipTracker's tools for automatic parameter selection (discussed in (Applegate and Danuser, 2010); maximum gap length, 16 frames; minimum sub-track length, 3 frames; search radius range, 5–10 pixels; maximum forward and backward angles, 30° and 15°; fluctuation radius, 5 pixels; maximum shrinkage factor, 1.5). A total of 2,591 sub-tracks were grouped into 991 compound tracks.

Tracking performance was first evaluated by comparing manually- and automatically-tracked growth phases of 19 MTs in a small peripheral region of the movie (Figure 9A, inset), where manual tracking of mCherry-tubulin labeled MTs was possible. The extensive overlap indicates excellent agreement (Figure 9B; compare also all computer-generated tracks with mCherry-tubulin labeled MTs in Supplementary Movie 9). Next we compared the full trajectories of the 19 MTs in detail. To do this, movies were generated for each MT showing side-by-side the manually-tracked trajectory and all the plusTipTracker-generated tracks in the same region over the same time period (Supplementary Movie 10; Figure 9C). The “Track Overlays” function was then used to select matching tracks and identify the corresponding track numbers. Finally, track profiles were compared visually as shown in Figure 9D. This procedure was used to fully annotate tracking performance for all 19 MTs (see “Track Comparisons” tab of Supplementary Excel spreadsheet).

While the agreement was generally very good, systematic differences did arise because of the different assumptions and limitations of the methods. For example, the human eye often perceived pauses when tracking the end of the MT, whereas the EB1 comet was actually still detectable, leading those phases to be classified as growth by plusTipTracker (Figure 9D, frames 10–11 and 26–27). Alternatively, because of the latency in comet formation (typically 2–4s (Thoma et al., 2010), i.e. in the present data 1–2 frames), short growth phases perceived by the eye in the tubulin-labeled channel were missed by the software (Figure 9D, frame 65). Finally, since growth phases must be at least three frames long to be generated by plusTipTracker, any growth phases shorter than this were missed (Figure 9D, frames 74–75, 84–85, 92–93, and 97). However, as can be seen in the inset in Figure 9C, these short events, which were classified based on the displacement of the MT end, did not necessarily correspond to the *bona fide* addition of tubulin subunits. While the molecular state of the MT is directly encoded in the image signal when tracking +TIP markers, the definition of growth, pause, and shrinkage in manual tracking greatly depends on the precision of the coordinate definition and the associated velocity thresholds used to classify growth and shrinkage events as significant.

Another common difference between the two methods arose from the fact that plusTipTracker cannot infer direct transitions from pause to shrinkage and vice versa, since each inference requires the presence of flanking growth phases. This led to the inference of higher pause or lower shrinkage speeds. Coupled with the lag time for comet formation (leading to slightly longer gaps) this effect caused some shrinkage events to be reclassified as pauses (e.g. MT 3, frames 68–75, where the reclassification is correct, and frames 79–84, where it is not).

As expected, plusTipTracker failed to capture full trajectories when two or more MTs ran parallel to one another at the same time. For example, in MT 15 two growth phases (frames 19–21 and 26–34) were missed because the comet merged with other comets (see Supplementary Excel spreadsheet). This created a 42-frame gap in tracking where manual tracking recorded pause/shrinkage (6–18), growth (19–21), shrinkage/pause (22–25), growth (26–34), and shrinkage (35–47). The software again captured the final hand-tracked growth phase (frame 48–58) and went on to correctly link to two more growth phases, extending the analysis by an additional 41 frames (Supplementary Movie 11).

While these analyses show some differences between the manual tracking of tubulin-labeled MTs and the dynamics inferred from automated tracking of +TIP proteins, the key question for most cell biological studies is whether these differences yield systematic deviations between the statistical distributions of the MT dynamics parameters. To test this we compared the results generated by plusTipTracker to the manually- and automatically-generated data set described in (Matov et al., 2010) (Supplementary Table 3). In (Matov et al., 2010) the bulk statistics of manually- and automatically-generated tracks were found to differ due to the unique assumptions and limitations of each method. Similar differences were also found with plusTipTracker, though certain parameters showed closer agreement with manual measurements, most notably pause durations and shrinkage speeds.

Manual tracking was prone to overestimation of growth and shrinkage rates as compared to automated tracking for several reasons. First, the manual method measured average frame-to-frame displacement, whereas the Matov method measured head-to-tail displacement (always a shorter value for the same number of frames). Although plusTipTracker measures frame-to-frame displacement, making it more similar in principle to manually-tracked data, growth speeds were still somewhat lower. Because of the centroid fitting used by the automatic detection approach, the noise in the comet coordinates was in the subpixel range, whereas the manual identification of MT ends in tubulin-labeled images afforded a precision of 1 pixel, at best. This noise was relatively high compared to the comet displacement between consecutive frames, resulting in an apparent increase of the instantaneous growth velocity in manual tracking. Also, the manual measurements contained many growth phases that were shorter in duration than the minimum number of frames used to generate growth tracks in the automatic method (four in Matov; three in plusTipTracker). Thus many short, fast growth phases measured in manual tracking were invisible to either automated approach. Further, for the frame rate and effective pixel size of the movie used for benchmarking, the minimum measurable frame-to-frame velocity by hand-tracking was $\sim 10 \mu\text{m}\cdot\text{min}^{-1}$ (1pix/frame). Both automatic trackers generated growth tracks slower than this rate, since growth was defined by comet detectability and not by speed. In contrast, the manual method classified these slow growth phases as pauses. These effects point to the need for a systematically-applied definition of growth and pause.

As for the difference in average shrinkage speed, in the Matov approach, pause-to-shrinkage transitions and vice versa were indistinguishable and therefore these phases were often averaged together, leading to much lower values ($17.6 \mu\text{m}\cdot\text{min}^{-1}$ compared to $39.4 \mu\text{m}\cdot\text{min}^{-1}$ with the manual approach). Moreover, the latency of comet formation after rescue reduced the inferred speeds. While plusTipTracker also has this limitation, the effect was ameliorated ($24.1 \mu\text{m}\cdot\text{min}^{-1}$) because of the more sophisticated bgap-detecting scheme. First, by introducing a circular search region around the termination point of a growth track (see Figure 5B), plusTipTracker explicitly distinguishes bgaps that are likely associated with shrinkage events from pausing events where the comet reappears by chance somewhat behind the termination point. In the Matov approach these latter events were counted as bgaps. As a result, in (Matov et al., 2010) the number of shrinkage events was much higher ($n=775$ vs. $n=105$ with plusTipTracker); the shrinkage speeds were underestimated ($17.6 \mu\text{m}\cdot\text{min}^{-1}$ vs. $24.1 \mu\text{m}\cdot\text{min}^{-1}$ with plusTipTracker and $24.9 \mu\text{m}\cdot\text{min}^{-1}$ with manual tracking); and the pause duration was too long (6.9 s vs. 3.3 s with plusTipTracker and 3.0 s with manual tracking) because many of the bgap assignments in the Matov approach that were converted to pause assignments with plusTipTracker were short-lived. The bias in (Matov et al., 2010) could be partially corrected by reclassifying slow bgaps as pauses (bgaps slower than $9.3 \mu\text{m}\cdot\text{min}^{-1}$). The threshold was determined by unimodal thresholding of the fgap velocity distribution, assuming that fgaps contained two populations, pauses and out-of-focus continuations of growth. Nevertheless, especially for short intervals of comet disappearance, random positional fluctuations may have led to quite fast apparent velocities,

preventing the reclassification. Therefore, the corrected data produced by the Matov approach still contained a substantially higher number of bgaps than the data generated by plusTipTracker, and the pause duration remained high. Second, in the Matov approach, all growth track initiations within a backward cone of 20° half-opening were considered candidate rescue events for the termination of a particular growth track termination. In plusTipTracker, the candidate rescue events are limited to a narrow band around the previous growth track, making the explicit assumption that MTs shrink and re-grow along their previous growth trajectory. Hence, plusTipTracker is more conservative in the search for rescue events. This results in a significantly reduced number of false positives, however at the expense of a higher number of false negatives. The statistics in Supplementary Table 3 suggest that the more restrictive model of plusTipTracker overall matches better with the data acquired by manual tracking.

Conclusions

We have introduced plusTipTracker, a user-friendly software package for the automated tracking, visualization, and analysis of MT dynamics in live cells using only +TIP markers of MT growth. Extensive validation of the general tracking algorithm used by the software package has been previously published (Jaqaman et al., 2008). Application of the sub-track linking concept to inferring MT pause and shrinkage events based on +TIP growth tracks has also been validated, using continuously labeled MTs (Matov et al., 2010). Validation is extended in this paper both to show improvements made by plusTipTracker and to point out that the sources of discrepancy between manual and automatic tracking are based on the different assumptions and limitations of each method.

Compared to these previous works, plusTipTracker employs an updated, more robust method for +TIP comet detection, which is self-adaptive and free of control parameters. The generic tracking framework in (Jaqaman et al., 2008) is adapted to specifically accommodate MT trajectories. Moreover, the concept of sub-track linking used in (Matov et al., 2010) is extended to account for MT curvature and integrated in the rigorous formalism for gap closing introduced in (Jaqaman et al., 2008). Thus, the plusTipTracker bundles state-of-the-art technology developed over the past several years in an integrated package, available to the community from <http://lccb.hms.harvard.edu>.

The package supports efficient image analysis of MT dynamics. In less than 10 minutes, hundreds to thousands of reconstructed tracks can be obtained from a single cell. Because of its graphical user interfaces, it requires minimal experience with Matlab for basic analyses, yet it is transparent to advanced users who wish to extend the functionality with application-specific readouts.

The many statistics calculated during post-processing must be interpreted with the assumptions of the tracking algorithm in mind. While forward and backward gaps obtained during the gap closing step do reflect the pausing and shrinking behavior of MTs, respectively, it is important to note that most parameters do not have a direct parallel in the traditional parameterization of MT dynamic instability via growth and shrinkage speeds and catastrophe and rescue frequencies (Matov et al., 2010; Thoma et al., 2010). The backward gap speed, for instance, seems analogous to shrinkage speed, but the two are not directly comparable because trajectory reconstruction by sub-track linking reveals only those shrinkage events followed by a rescue within a highly constrained spatial and temporal window. By construction, the algorithm misses unrescued shrinkage events. Furthermore, due to latency in the assembly of a detectable comet, the measured rescue events are delayed, leading to an additional underestimation of the actual shrinkage speed. While the statistics calculated by plusTipTracker should not be compared to those obtained by manual

tracking of fully-labeled MTs, differences in these parameters across experimental conditions do in fact reveal rich information about differences in MT regulation in different molecular backgrounds (Matov et al., 2010; Myers et al., 2011; Thoma et al., 2010; Wu et al., 2011).

plusTipTracker's ability to infer MT pause and shrinkage states, coupled with its capacity to directly measure fine shifts in MT subpopulations, promises to yield mechanistic insight into the spatial regulation of MTs by MAPs, signaling proteins, and drugs across many cell types and even phases of the cell cycle. In addition, the speed and robustness of the processing make possible for the first time a high-content screen of MT cytoskeleton dynamics in live cells.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Abbreviations used

EB3	end-binding protein 3
LAP	linear assignment problem
MT	microtubule
+TIP	MT plus end binding protein
ROI	region of interest

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Figure 1.

+TIPs mark growing MT plus ends. (A) Dual-channel image of tdTomato-EB3 (red) and GFP-tubulin (green) taken with a spinning-disk confocal microscope (courtesy of Ken Myers, NIH/NHLBI). Bar, 10 μ m. (B) +TIPs have a higher binding affinity for growing MT plus ends than for the MT lattice, leading to the comet-like appearance observed in fluorescence images like (A). (C) plusTipTracker first tracks +TIP comets (red ovals) to form growth sub-tracks (I, III, V; solid black lines) and subsequently groups sub-tracks inferred to have come from the same MT to reconstruct shrinkage (II; dashed black line) and pause (IV; dashed black line) behavior. Transparent red ovals indicate newly-formed comets. The time lag between MT rescue and reappearance of a detectable comet can lead to underestimation of shrinkage speed and positional drift during pause.

Figure 2. plusTipTracker user interfaces. (A) Interface for project setup, detection, tracking, and post-processing steps of analysis. Red box indicates a blow-up of the interface to the detection algorithm. As a default the software operates with a watershed-based comet detection (see Methods). (B) Interface for various types of track visualization and further analysis, including: interactive track overlays ('Track Overlays'); movies of regions or individual tracks ('Track Movies'); movies of tracked comets color-coded by speed ('Speed Movies'); sub-cellular region-of-interest selection ('Sub-ROIs'); and MT subpopulation analysis ('Quadrant Scatter Plots'). See (Applegate and Danuser, 2010) for details.

Figure 3. Particle detection by watershed-based method. (A) Raw image of EGFP-EB3. Bar, 5 μ m. (B) Intensity landscape of the Difference-of-Gaussian image derived from the white-framed inset shown in (A). (C) Idealized intensity landscape illustrating detection algorithm. Decreasing thresholds, represented by the three horizontal lines crossing the peaks, generate the peak contours shown in panels (i), (ii), and (iii). The contours for peaks 1 and 2 are retained from the middle threshold, while the contour from peak 3 is retained from the lowest threshold. The final detection result is shown in the red-framed inset. (D) Manual (blue) and automatic (red) detection of particles from the image in (A). False positive (yellow) and false negative (cyan) rates are $100(1-99/102) = 2.9\%$ and $100(1-99/103) = 3.9\%$, respectively (see text for formula details). (E,F) False positive and false negative detections, as a function of the comet signal-to-noise ratio (SNR, defined as amplitude of comet signal divided by standard deviation of the background intensity) and comet eccentricity. Red asterisks indicate the parameter settings used to generate Supplementary Movie 2.

Figure 4.

Particle detection by anisotropic Gaussian model fitting method. (A) User interface to the detection algorithm when the option 'Gaussian fit' is selected. See Methods for an explanation of the parameters. (B,C) Comparison of the performance of the watershed method and the anisotropic Gaussian model fitting method in detecting particles representing substantially elongated comets. The watershed method tends to detect multiple particles per comet, while anisotropic Gaussian model fitting explicitly takes into account the anisotropy of the signal. Detection performance on the entire time-lapse sequence is illustrated in Supplementary Movie 3.

Figure 5.

Tracking and inference of complete MT plus-end trajectories. (A) Sub-tracks, which are generated by linking +TIP comets and which indicate the growth of MTs, are unidirectional and often collinear (arrow pairs). Pairs of collinear growth sub-tracks may become candidates for sub-track linking. Bar, 5μm. (B) Illustration of the candidate selection for sub-track linking. Sub-track i (black) starts at $t = 0$ and ends at $t = 8$. Candidate sub-tracks j (blue and green) for linking to sub-track i must initiate in the gray search region and start within Δt_{max} frames (user input to the tracking software) from the end-point of sub-track i . Candidates chosen from the light gray region will generate inferred shrinkage events (blue), while candidates chosen from the dark gray region will generate inferred pause or continuation-of-growth events (green; the latter arises due to detection failure or the comet moving temporarily out of focus). The cost of linking depends on the time gap between the sub-track end and candidate start, Δt_{gap} , and the three spatial parameters $d_{||}$, $d_{\perp}$, θ . See text for details of these parameters and the cost function. (C) The cost of sub-track linking is directionally unbiased, as shown by the distribution of costs in the forward (green) and backward (blue) directions for all potential links (left). For tracks with only one candidate, the balance is maintained (right). Sub-track pairs with costs higher than the death cost (vertical line) will not be chosen for linking, allowing proper termination of trajectories

where pause or shrinkage is unlikely. (D) The 'Track Overlays' tool (Figure 2B) was used to show all tracks on the image (left; bar, 10 μ m), zoom in, select an individual track (right; bar, 5 μ m) from the hundreds shown in the inset, and view its profile (table). In this example profile the lifetimes and displacements are reported in seconds and microns, while the software returns them in frames and pixels. This track corresponds to Supplementary Movie 4. (E) Evaluation of frame-to-frame and sub-track linking (forward and backward gaps separated) in simulated +TIP movies.

Figure 6. Spatial maps of MT dynamics. (A) Top row: stacked speed, lifetime, and displacement distributions for growth, fgap, and bgap sub-tracks. Rows 2–4: growth, fgap, and bgap sub-tracks color-coded by speed (left column), lifetime (center column), and displacement (right column). Bar, 10 μm . (B) Initiation (top row) and termination (bottom row) sites for fgaps (left) and bgaps (right). Merged images are shown in the right column. The merged distributions of fgap and bgap initiation/termination sites are equivalent to the distributions of termination/initiation sites of growth events participating in compound trajectories.

Figure 7. Sub-cellular organization of growth speed. (A) Using the ‘Sub-ROIs’ tool (Figure 2B), growth sub-tracks were extracted from five regions of the cell (center, blue; front, green; back, yellow; left, red; right, magenta). Bar, 10 μ m. (B) Growth speed distributions for the five regions. (C) Discrimination matrix containing the results of two statistical tests for each pair of cell regions. Below the diagonal: percent confidence that the distributions are different, from a bootstrapped, mean-subtracted Kolmogorov-Smirnov test. Above the diagonal: p-values for a permutation t-test of the means. Significant differences shown in gray.

Figure 8.

Proportions of MT subpopulations classified by growth speed and growth lifetime. (A) The 'Quadrant Scatter Plot' tool (Figure 2B) was used to generate a speed vs. lifetime scatter plot split into quadrants based on mean growth speed ($21.7\mu\text{m}\cdot\text{min}^{-1}$; corresponding to the 49th percentile) and mean growth lifetime (12.9s; corresponding to the 66th percentile), generating four distinct subpopulations of growth sub-tracks. (B) Sub-tracks corresponding to the data points in (A) are overlaid together on the image with the same color scheme to show relative spatial arrangement. The four subpopulations thus represent slow and long-lived (C), fast and long-lived (D), slow and short-lived (E), and fast and short-lived growth events. Growth sub-tracks extracted from the sub-ROIs used in Figure 7(G) show the relative proportions of the subpopulations in different sub-cellular regions (H).

Figure 9. Evaluation of plusTipTracker performance on two-color dataset. (A) Overlay of plusTipTracker results on EB1-EGFP image. (B) Growth tracks obtained by manual and automatic tracking overlaid on mCherry-tubulin image corresponding to inset in (A). (C) Visual comparison of manual (left) and automatic (right) tracks for a single MT (No. 1 in the annotated list; see Supplementary Excel file), overlaid on two-color merged image. Blue crosses represent hand-tracked comet positions. (D) Annotated track profiles describing sources of variation in manual and automatic tracking results.