SUPPORTING MATERIAL

Interactive, computer-assisted tracking of speckle trajectories in fluorescence microscopy: application to actin polymerization and membrane fusion.

Matthew B. Smith[1], Erdem Karatekin[2,3], Andrea Gohlke[3], Hiroaki Mizuno[4], Naoki Watanabe[4], Dimitrios Vavylonis[1,*]

[1] Department of Physics, Lehigh University, Bethlehem PA 18015
[2] Centre National de la Recherche Scientifique and Université Paris Descartes, UMR 8192, Centre Universitaire des Saints-Pères, 45 rue des Saints-Pères, Paris, France
[3] Department of Cell Biology, School of Medicine, Yale University, New Haven CT 06520
[4] Laboratory of Single-Molecule Cell Biology, Tohoku University Graduate School of Life Sciences, Sendai, Miyagi 980-8578, Japan

* Correspondence: vavylonis@lehigh.edu

1. Additional Information on Particle Detection Methods

"Locate Speckles," has three parameters: threshold, size, and minimum distance. Using the threshold value, a binary image is generated. A two-pass connected components algorithm (1) is then applied to find speckle mark candidates. The position of a candidate is the center of mass of the connected component. Candidates are then removed if the number of pixels of the connected component is less than the size parameter. If the distance two candidates is smaller than the minimum distance parameter, the candidate with the smaller size is removed.

"Template Locate," performs the same operation as the "Locate Speckles" method except that it uses existing speckle marks to create a Normalized Cross Correlation (NCC) (2) filtered copy of the image. The NCC template is made by averaging a square region of adjustable size centered at existing speckle marks. Thus the program can be trained simply by clicking to define a template.

2. Additional Information on Tracking Models

"Static." The Static Model places speckle marks on all frames preceding the first frame of the speckle track, at the position of the first speckle mark. In a similar fashion, it places speckle marks on all frames following the last mark of the track at the position of the last mark.

"Extend Linear Refine." The Extend Linear Refine Model is similar to the Static model but it additionally tries to account for a small constant velocity during the movie. In this model, each additional mark at the beginning or the end of the speckle track is adjusted using the "center of intensity" algorithm described in Eq. (1) below (around the position of the first or last existing

mark, respectively). Once a speckle track that spans the whole movie is constructed, this model performs a weighted least squares fit to positions of the speckle track. Each point is weighted proportionally to the local intensity to obtain a velocity. Finally, all marks are updated to lie on a straight line trajectory determined by this velocity.

"Constant Velocity NCC" model is the same as Diffusing NCC but the search for the best candidate occurs over a square whose center is displaced from the position of the previous speckle mark. To estimate *v*, a linear least squares fit on each existing speckle track is performed during initialization. The velocities from the fit are averaged to calculate *v*.

3. Additional Information on Refine Position Models

Speckle tracks can be refined to improve the position of existing speckle marks. A technique used to refine positions moves the speckle mark to the center of intensity of an area of size 5x5 pixels:

$$\Delta x = \frac{\sum_{i=-2}^{2}\sum_{j=-2}^{2} I(x+i, y+j)(x+i)}{\sum_{i=-2}^{2}\sum_{j=-2}^{2} I(x+i, y+j)}, \quad \Delta y = \frac{\sum_{i=-2}^{2}\sum_{j=-2}^{2} I(x+i, y+j)(y+j)}{\sum_{i=-2}^{2}\sum_{j=-2}^{2} I(x+i, y+j)}. \qquad (1)$$

This process is applied iteratively until the change in position is small (less than 0.01 px) or five iterations have occurred. The background noise will have a center at the center of the square but by iterating, the bright feature will 'pull' the center towards it.

The "Adjustment Model" modifies existing speckle tracks by using the above refine technique for every speckle mark. The "Refine Model" performs the operation of the Adjustment Model, then it applies a least squares fit to the positions of the existing marks as in the Extend Linear Refine Model. It also fills in missing speckle marks between the first and last frame of the speckle track.

The "Gaussian Fit" model refines the position of speckles with sub pixel accuracy. It fits a 2D Gaussian to the intensity of a 11x11 px^2 square near a speckle mark as follows using a least squares fit. The fit is started with a user-defined standard deviation of the Gaussian, σ (1 px is the default value). The position of the center of the Gaussian is varied using a variant of the simplex method as follows. Four points are picked along the x and y axes with a distance of 0.75 px from the originally-estimated position. The amplitude and background of a 2D Gaussian centered at each point is found using a linear least squares fit, and the error is calculated. The results are sorted according to the error, which is the sum of the squares of residuals. The point with the highest error is moved toward the point with the lowest error, at the midpoint position. The process of moving the point with the highest error is iterated and continues until the largest difference in error among the points is below a threshold value (10^{-6}). This results in an estimate of the best position. The whole process is repeated for different σ, using a 1D golden section algorithm with initial step $d\sigma = 0.1$ px (3) to select for the sigma that minimizes the error. The fit is finished when the change $d\sigma$ is sufficiently small (less than 0.02 px). While this is perhaps

the most accurate model when the particles are Gaussian spots, it is also the most computationally intensive.

4. User Interface

Users can manually create and modify speckle tracks by clicking on the image (Fig. S1A), and by moving, trimming and merging of tracks. Further tools to aid in the user's judgment are the Profiler, the Selection Table and the Reslice Control. The Profiler (Fig. S1C) graphs the intensity of the speckle over a circle with user-adjustable radius, r_{in}, and over an annulus with inner radius r_{in} and outer radius, r_{out}. The Selection Table (Fig. S1B) shows speckle track values such as maximum displacement per frame and distance of closest approach to neighboring tracks. The table allows users to sort and select speckle tracks, navigate the image stack and find problematic cases. The Reslice Control (Fig. S1D) makes a *y*-*t* projection of the original image stack to facilitate viewing the intensity through time.

5. Tracking Precision

To evaluate the accuracy of our Gaussian Fit and tracking algorithm, we used a previously described method (2, 4) . Simulated particles that were stationary during exposure were displaced by a small distance (0.27 px) between exposures. We generated images of 12 such simulated particles for 101 frames using the method described in the "Single Molecule Diffusion – Simulations" section of the main text. To check the effect of pixel size, λ, the intensity of each particle was convolved with a Gaussian kernel of standard deviation σ = 0.5, 1, or 1.5 px. To study the effect of STN we varied the standard deviation, σ_N, of the added Gaussian noise.

Here, we define the signal to noise to be STN = I/σ_N where I is the average intensity (above the background) at the position of the speckle mark. To better compare with previous studies we divide with σ_N instead of the standard deviation of the noise at the position of the particle (as was done in Table 1): otherwise fluctuations in the distribution of particle intensity among the pixels near the particle position leads to an absolute maximum STN value, even without added noise.

For each set of images, speckles were seeded, and tracked automatically using the Constant Velocity NCC model followed by the Adjustment and Gaussian Fit models. Fig. 1 shows the dependence of the precision, ε, equal to the standard deviation of the difference between particle position and speckle mark, on STN and σ/λ. We find ε scales approximately linearly with σ/λ and 1/STN, as in other algorithms (5, 6). The magnitude of our precision is comparable to those of previous tracking studies (2, 4), with small differences that are likely due to small differences in STN calculations and type of noise (the authors of (4) simulated Poisson noise). Because our tracking algorithm scans a large region of space before placing a speckle mark, the "bias" (2, 4), i.e. the average distance between speckle mark and particle position, was negligible.

6. Additional Information on Single-molecule Imaging of Fluorescent Actin and Capping Protein

Live cell imaging was carried out as described (7). Cells were transiently transfected using Superfect (Qiagen) and maintained after passage into fresh flasks. Before experiments, cells were trypsinized and allowed to spread on a poly-L-lysine (PLL)-coated glass coverslip attached to a flow cell in 70% L-15 medium without serum for 30-60 min. The flow cell was then placed on the stage of an Olympus BX51 microscope equipped with Cascade II:512 (Roper Scientific). Fluorescent speckle microscopy was carried out by observing cells expressing a low amount of EGFP-tagged proteins. A restricted area near the cell edge was illuminated using a 75 W xenon illumination system. Imaging acquisition was carried out at 21-23 °C using the Metamorph software (Molecular Devise) and Olympus oil objectives, PlanApo 100× (NA 1.40) or 150× (NA 1.45).

7. Additional Information on Single-vesicle Docking and Fusion Experiments

The following lipids were purchased from Avanti Polar Lipids, Inc. (Alabaster, Alabama): *1,2-dioleoyl-sn-glycero-3-phosphocholin*e (DOPC), *1,2-dioleoyl*-sn-*glycero-3-phospho-L-serine (sodium salt)* (DOPS), *1,2-dioleoyl*-sn-*glycero-3-phosphoethanolamine-N-[methoxy(polyethylene glycol)-2000] (ammonium salt)* (mPEG2000PE), *1,2-dioleoyl*-sn-*glycero-3-phosphoethanolamine-N-(7-nitro-2-1,3-benzoxadiazol-4-yl) (ammonium salt)* (NBD-PE), *1,2-dioleoyl*-sn-*glycero-3-phosphoethanolamine-N-(lissamine rhodamine B sulfonyl)* (ammonium salt) (LR-PE). Expression, purification and reconstitution of the synaptic/exocytic vesicle-associated v-SNARE (*soluble N-ethylmaleimide-sensitive factor attachment protein receptor*) proteins VAMP2/synaptobrevin and the target membrane associated t-SNAREs syntaxin and SNAP25 were described previously (8). Reconstitution of the SNARE proteins into small unilamellar vesicles (SUVs) was also done following ref. (8) using the following lipid composition, in mole %: DOPC/DOPS/LR-PE/mPEG2000K =79.2/15/0.8 for the v-SNARE vesicles (v-SUVs) and DOPC/DOPS/NBD-PE/mPEG2000K=79.5/15/0.5/5 for the t-SNARE containing supported bilayers (t-SBLs). To have a relatively clean background, in this study we reduced the LR-PE label density on the v-SUVs to 0.8 mole %, whereas 1-2 % were used previously (8).

Planar, supported bilayers decorated with t-SNAREs were made to cover the bottom of microfluidic channels by bursting and fusion of t-SUVs onto clean, hydrophilic glass coverslip substrates. The NBD-PE label in the supported bilayer is used to assess the fluidity and quality of the t-SBL before introducing the v-SUVs into the channel.

The microscopy setup, the formation and characterization of the t-SBLs are described in ref. (8). Importantly, two modifications here allowed us to detect single fluorescent lipids to be detected in the SBL after fusion for the first time: (i) to reduce the background signals, we reduced the LR-PE label density on the v-SUVs to 0.8 mole %, whereas 1-2 % were used previously (8), and (ii) we used total internal reflection fluorescence microscopy (TIRFM) instead of far-field epifluorescence that was employed previously, allowing image acquisition at 31 frames/sec full-frame (512x512 pixels) or at 57 frames/s from a 400x256 pixel region of interest using a back-thinned EM-CCD camera (iXon DU897E, Andor technology). We used custom-made, high

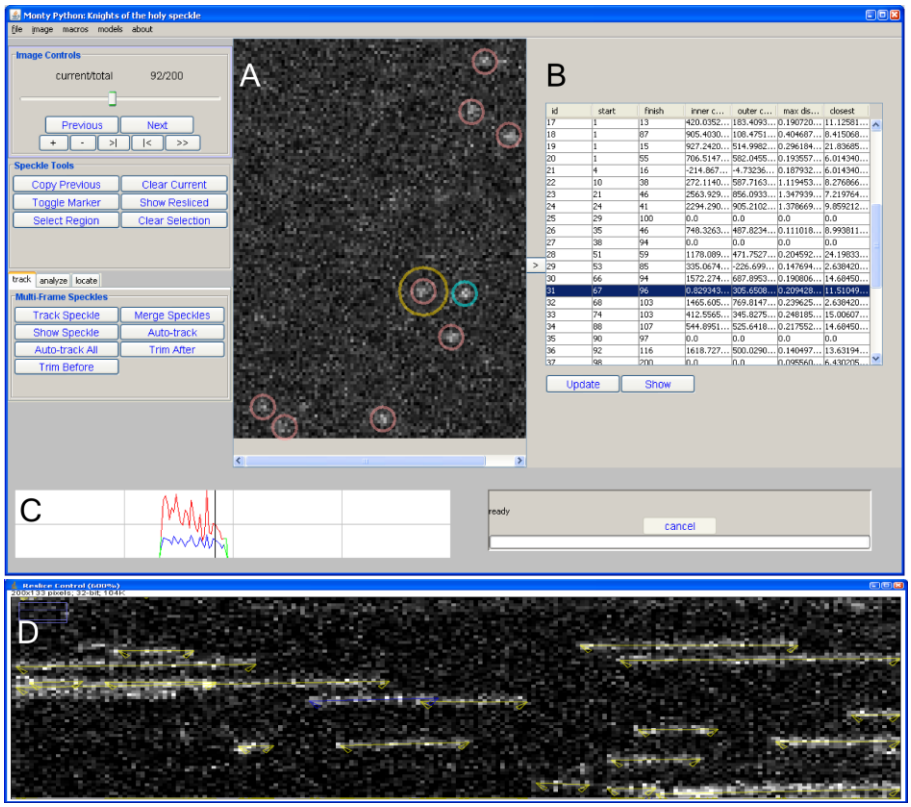
quality filters (clean-up: zet532/10x, dichroic: zt532rdc on custom 2 mm thick substrate, emission: hhq545lp and et605/70m) from Chroma Technology Corp. (Bellows Falls, VT). The fastest acquisition rates here are about 7 times faster than in ref. (8).

1. Acharya, T., and A. Ray. 2005. Image Processing: Principles and Applications. Wiley-Interscience.
2. Cheezum, M. K., W. F. Walker, and W. H. Guilford. 2001. Quantitative comparison of algorithms for tracking single fluorescent particles. Biophys J 81:2378-2388.
3. Press, W. H., S. A. Teukolsky, W. T. Vetterling, and B. P. Flannery. 2007. Numerical Recipes: The Art of Scientific Computing Cambridge University Press, Cabmridge.
4. Sbalzarini, I. F., and P. Koumoutsakos. 2005. Feature point tracking and trajectory analysis for video imaging in cell biology. J Struct Biol 151:182-195.
5. Crocker, J., and D. G. Grier. 1995. Methods of Digital Video Microscopy for Colloidal Studies. Journal of colloid and interface science 179.
6. Savin, T., and P. S. Doyle. 2005. Static and dynamic errors in particle tracking microrheology. Biophys J 88:623-638.
7. Miyoshi, T., T. Tsuji, C. Higashida, M. Hertzog, A. Fujita, S. Narumiya, G. Scita, and N. Watanabe. 2006. Actin turnover-dependent fast dissociation of capping protein in the dendritic nucleation actin network: evidence of frequent filament severing. J Cell Biol 175:947-955.
8. Karatekin, E., J. Di Giovanni, C. Iborra, J. Coleman, B. O'Shaughnessy, M. Seagar, and J. E. Rothman. 2010. A fast, single-vesicle fusion assay mimics physiological SNARE requirements. Proc Natl Acad Sci U S A 107:3517-3521.

		u-track		Particle Tracker		Speckle TrackerJ	
D ($\mu m^2/s$)	STN	N_{tracks}> 20 frames	Mean track length (frames)	N_{tracks}> 20 frames	Mean track length (frames)	N_{tracks}> 20 frames	Mean track length (frames)
0.01	22.6	12	301	11	301	12	301
0.01	4.4	12	301	22	96	12	301
0.1	20.4	14	247	12	278	11	299
0.1	3.7	14	251	6	24	12	289
1	6.3	20	174	30	109	13	274
1	2.9	46	75	16	26	29	118
4	3.4	47	29	12	27	10	144
4	2.1	24	29			5	101

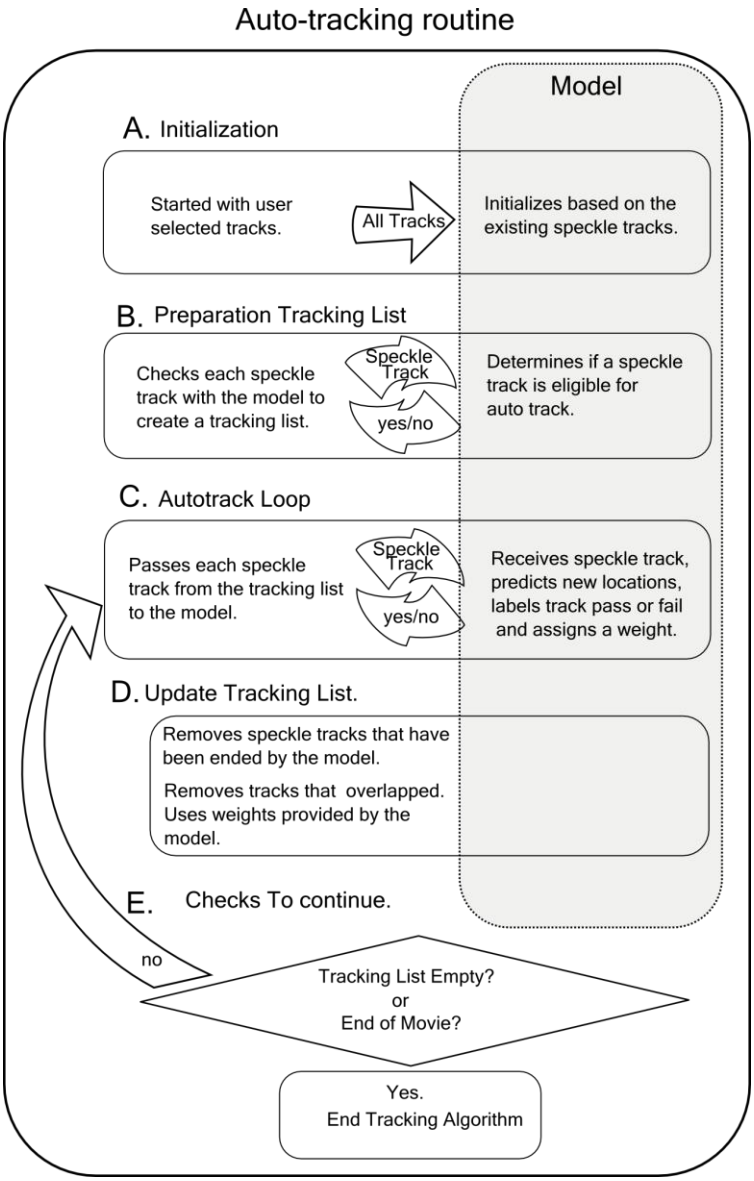
Table S1. Results of tracking particles in simulated images using three different software tools. First two columns show simulated diffusion coefficient and STN value. Each movie had 12 particles and was 301 frames long. The table shows the number of particle tracks longer than 20 frames and mean track length from the runs we used to calculate the diffusion coefficients in Table 1. At low STN, bits of the same particle trajectory appear as different tracks as particles are lost and found, so the number of tracks is more than 12 in many cases. For Speckle TrackerJ we switched from batch auto-tracking to a combination of auto-tracking and manual interaction at D = 4 $\mu m^2/s$. Empty boxes: we were unable to find good tracking parameters.

Figure S1



Speckle TrackerJ, user interface. (A) Image with marked speckles. (B) Selection Table shows data about tracked particles; it can be used to select speckle tracks. (C) Profiler shows the selected speckle intensity in different frames. (D) Reslice Control shows a projection of the movie with time as horizontal axis and the start and end points of speckle tracks.

Figure S2



Flow chart of automated tracking algorithm. Actions in the grey region are performed by the model and all other actions are performed by the tracking algorithm.