Supplemental Materials

Molecular Biology of the Cell

Mangeol et al.

Supplementary information

KymographClear and KymographDirect: two tools for the automated quantitative analysis of molecular and cellular dynamics using kymographs

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KymographClear

Fourier filtering

The principle behind this feature is demonstrated in Figure S1. In the Fourier transform of a kymograph, forward-moving, backward-moving and pausing (or static) components are located in different quadrants (Figure S1A-C), which allows straightforward separation. Images reflecting only forward motion, backward motion or pauses can be obtained by masking the Fourier-transformed kymographs (setting the pixel values of two diagonally opposed quadrants to zero), followed by inverse Fourier transformation. An additional benefit is that the noise is reduced, by a factor of two in case two diagonally opposite quadrants are used as a mask. In most cases, diffusing particles show up in the same region of the Fourier-transformed images as where pausing particles show up (along the horizontal, Figure S1B). Thus, Fourier filtering (of static components) can also be used to extract diffusing components. The geometry and dimensions of the spatial filter used (the main diagonals) limit the maximum displacement of diffusive particles to one pixel per frame. In Figure S1C the efficacy of kymograph Fourier filtering to discriminate pausing, forward and backward-moving objects is demonstrated.

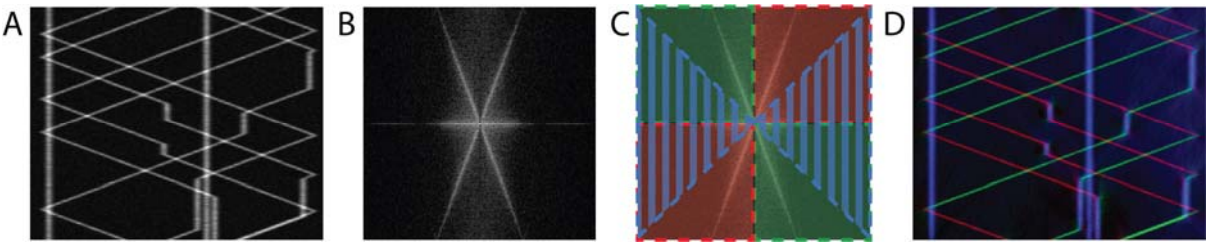

Figure S1. Effect of Fourier filtering on a kymograph. (A) Simulated kymograph displaying forward motion (lines traced from top left to bottom right), backward motion (lines traced from top right to bottom left) and pausing pools (vertical lines). (B) Absolute value of the Fourier transform of kymograph (A). (C) Same as (B) with the regions containing information on forward-moving (red), backward-moving (green) and pausing (blue stripes) components highlighted. (D) Kymograph with forward-moving, backward-moving and pausing components extracted using Fourier filtering and displayed in red, green and blue, respectively.

Fourier filtering can cause ringing artifacts at the edge of an image. The solution we applied to overcome this problem is to Fourier transform an image paved with the kymograph as depicted in

Figure S2, where each edge of the kymograph is matched with a corresponding copy properly oriented. The algorithm used in *KymographClear* applies a Fourier transform on this paved image, masks the Fourier-transformed image, applies an inverse Fourier transform and selects the kymograph in the center of the resulting paved image.

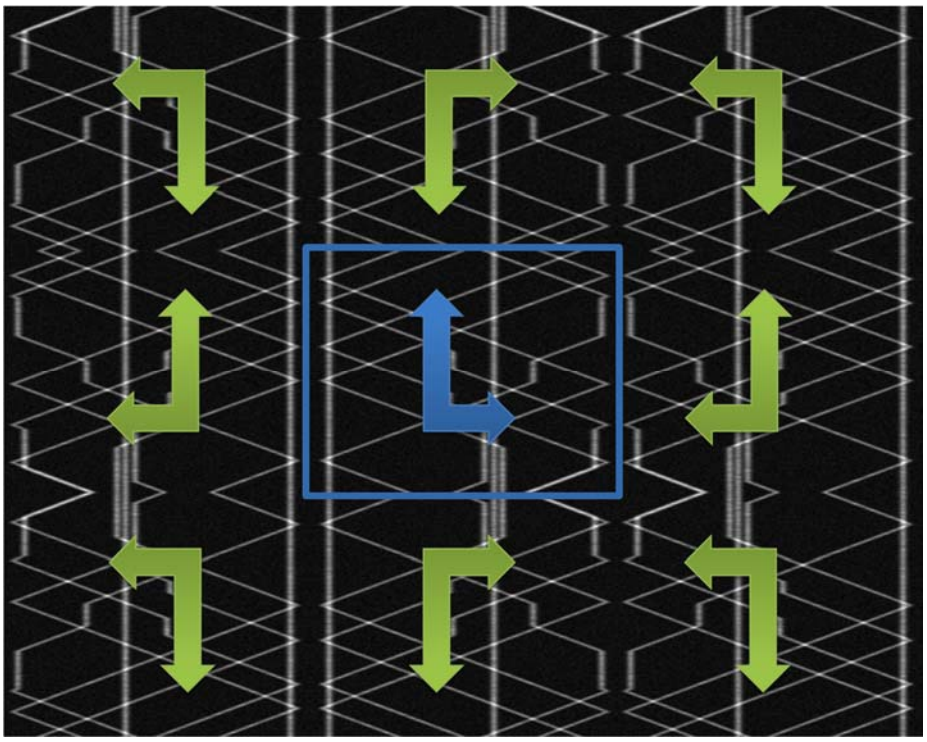

Figure S2. In order to reduce ringing effects caused by Fourier filtering, a Fourier transform is applied on an image paved with nine kymographs oriented as indicated. After filtering only the center kymograph (blue frame) is retrieved.

Background evaluation.

For image sequences obtained with fluorescence microscopy, it can be desirable to correct the signal amplitude for background. Because of potential non-uniformity across the image, background correction needs to be performed carefully. In our program the background is evaluated in a user-selected region of interest for each frame and can be use at later stages in *KymographDirect*.

KymographDirect

Background and photobleaching correction.

Before the kymographs can be analyzed, they might need to be corrected for background and photobleaching of the particles of interest. Background signals due to out-of-focus fluorescence light, autofluorescence of the sample (i.e. fluorescence emerging from different sources than the added fluorescent label), scattered excitation light or electronics, result in unwanted offset of the measured fluorescence intensity. The user has the option to correct kymographs for background. To this end, use is made of the stored average pixel intensity values of a region next to the track of interest (as described in *KymographClear* section). Typically, the background intensity decreases over time due to photobleaching, which affects some of the background sources. In our experience, the time evolution

of the background signal can be well described by an exponential decay with offset. Our background-correction algorithm performs a fit with such a function to the background signal and subtracts the fitted values from the kymograph.

Another aspect the data needs to be corrected for is photobleaching, the gradual decrease of the fluorescence intensity of the particles of interest. To correct background-corrected kymographs for photobleaching, we divide the kymograph by a correction function. This function is obtained from the kymograph by fitting the position-averaged intensity as function of time with an exponential decay. This correction is optional and should be applied only in situations where the number of fluorophores is constant.

Fourier filtering

Discrimination of forward-, backward-moving and pausing (or slowly diffusing) components is performed in the same way as described for *KymographClear* except that in *KymographDirect* background and photobleaching-corrected kymographs are used (when selected by the user). In later stages of the analysis, the program only considers the user-selected direction of motion.

Tracking trajectories

The linear motion of a particle results in a (curved) line in a kymograph, with slope directly related to its velocity, a trajectory. The tracking section of *KymographDirect* allows for the automated extraction of trajectories. In brief, the tracking algorithm operates as follows: first, the average velocity is estimated for each position on the track; second, particles are detected and their trajectories in the kymograph are determined. The fidelity of tracking is substantially increased by making use of the estimate of the local particle-averaged velocity. In case of pause or slowly diffusing particles, this step is simplified as the average velocity is set to zero.

Estimating the local, particle-averaged velocity.

To estimate the average velocity at a given position, we chose to correlate the intensity information between adjacent vertical pixel lines in the kymograph, corresponding to adjacent positions on the track. Direct cross-correlation of the intensity between lines has been performed before (Welzel *et al.* 2009), but in our experience does not lead to robust results, because of image noise and signal variation. Our algorithm follows the following sequence:

- Vertical kymograph lines (i.e. intensity as a function of time) are low-pass filtered to decrease noise (Figure S3A).
- Peaks are detected with sub-pixel (time) resolution in these vertical kymograph lines, using an intensity threshold set by the user.
- The peaks in the vertical (time) kymograph lines are replaced by identical, normalized triangular functions centered at the detected peak position (Figure S3B-C).
- Cross-correlation between two successive vertical pixel lines intensity signals obtained in the previous step: this gives an estimate of the average time particles need to translate the width of a pixel along the track, which can be converted to a local, particle-averaged velocity.
- Finally, to improve the robustness of the algorithm, the location-dependent velocity is low-pass filtered and fitted with a third order polynomial, which is the final output of this step (Figure S3D).

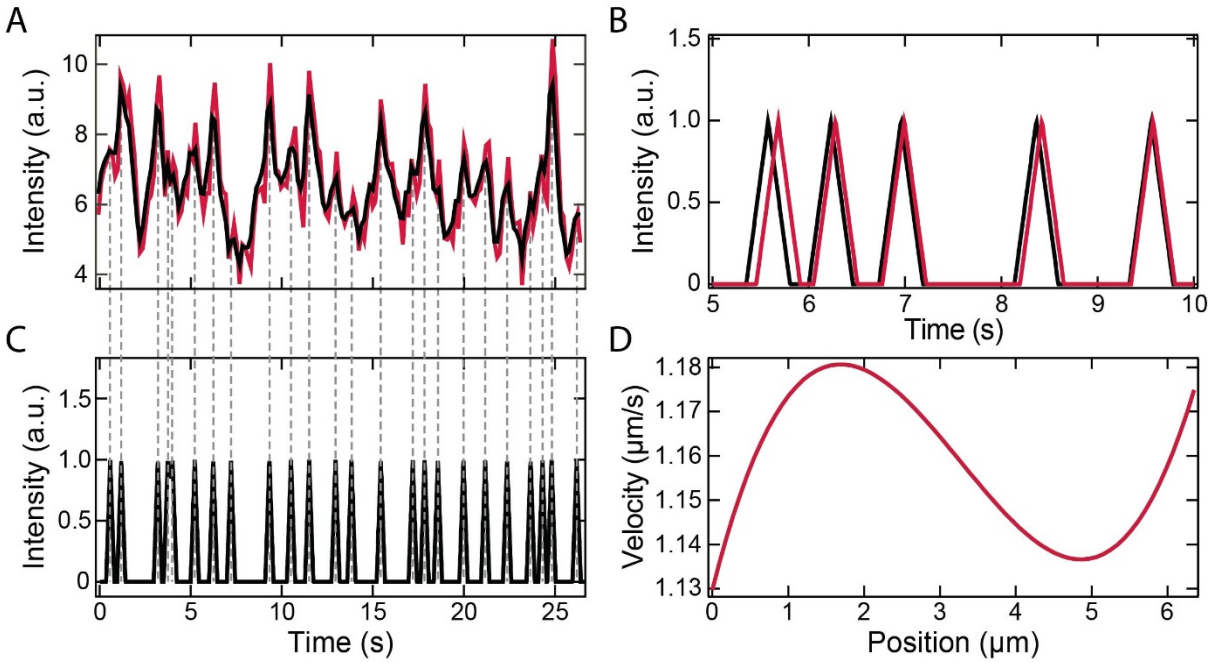

Figure S3. Evaluation of average local velocity in a kymograph. (A) Example of an intensity versus time signal on a given position as obtained from Figure 9B kymograph (red) and the same signal processed by the first order low pass filter of the algorithm (black). (B) At each position of a peak found in the filtered signal, the algorithm places a triangular function of fixed height and width. (C) Example of two signals transformed into triangular functions obtained from adjacent pixel positions (black position n, red position n+1); the delay observed between these two signals signal is evaluated by autocorrelation. (D) Average velocity versus position of Figure 9B kymograph as obtained by the algorithm.

In case particles move very slowly, less than one pixel per ten frames, the cross-correlation algorithm as described above fails. Local velocity can then be obtained robustly by using the same algorithm on 90 degrees rotated kymographs. This version of the algorithm can be selected by the user, when low velocities are expected. As an alternative, the diffusive-particles mode can be selected.

Detecting single trajectories.

Single trajectories are extracted as follows: points potentially belonging to trajectories are detected on the kymograph by peak detection; the algorithm then connects the points using an algorithm that search for the most likely displacement based on the average velocity obtained in the previous step. In more detail, the algorithm to extract individual trajectories consists of the following steps:

- Peaks are automatically detected in the vertical (position constant) lines of the kymograph obtained previously with sub-pixel (time) resolution, using an intensity threshold set by the user. For low signal-to-noise ratio data, the kymographs can be filtered for noise (along the time axis) prior to peak detection; in the program this mode is named “noise reduction”. To this end, a line of interest (along the time-axis) is first summed with the two lines directly adjacent. This sum is filtered with a first-order low-pass filter, in the increasing time direction and in the opposite direction, and the two resulting datasets are summed.

In diffusive-particle mode, the algorithm is modified: peak detection and filtering are performed on horizontal lines instead.

- Then, the algorithm selects the highest intensity peak in the first vertical line (x=0) and searches for a peak belonging to the same trajectory in the neighboring kymograph line, within a window set by the location-dependent velocity obtained in the previous step in the program (Figure S4). This part of the algorithm works as follows. Given a peak position x_0 at time t_0, the peak in the neighboring line (at position $x_0 + 1$) is allowed to be located between time points $t_0 + 1/((\alpha+1)V)$ and $t_0 + (\alpha+1)/V$ (*i.e.* instantaneous velocity of the particle is allowed to lie between $(\alpha+1)V$ and $V/(\alpha+1)$, with V the average velocity of particles at position $x_0 + 1$ and α a factor that can be chosen by the user between 0 and 10. In other words, α sets the width of the search window: for kymograph with low variability in velocity, α can be set close to 1 (as used for Figure 9); when velocity variability is higher, a larger value of α will increase the fraction of trajectories detected (in Figure 10, an α of 6 was used). In case multiple peaks are found within this window, peaks are weighted with the function $((t-(t_0+1/V))+1)\exp(-(t-(t_0+1/V))-1)$, which is a skewed bell function with a maximum centered on the most probable peak, i.e. $t_0 + 1/V$. The peak with the highest weight is selected for further analysis.
  When the diffusive-particle mode is selected, the analysis is modified. The algorithm selects the highest intensity peak in the first horizontal line (t=0) and searches for a peak belonging to the same trajectory in the next frame (t=1). If x_0 is the first peak detected, the next peak is searched in the next frame for positions $x_0 - \alpha$ and $x_0 + \alpha$; here α is chosen between 0 and 10. In case multiple peaks are found within this window, peaks are weighted with a triangular function centered around x_0. The peak with the highest weight is selected for further analysis.
- In case no peak is found in the previous step, the algorithm looks for peaks in the next four vertical pixel lines (positions), in the same way as the previous step. At position $x_0 + n$, the eligible peak is allowed to be located between time point $t_0 + n/((\alpha+1)V)$ and $t_0 + (\alpha+1)n/V$.
  In diffusive-particle mode, the algorithm looks for peaks in the next four horizontal lines (frames), in the same way as the previous step. If the first peak is found at frame 0 at position x_0, the next peak in frame n is allowed to be located between the positions $x_0 - \alpha n$ and $x_0 + \alpha n$.
- In case a peak is found that is connected to the previous one, the algorithm looks for a connected peak in the subsequent kymograph position line and so on, constructing the trajectory point by point.
- In case no further peak is found, the positions of the peaks found are gathered into a segmented line.
- Then, the algorithm repeats this process, using peaks that have not been used so far, until there is no peak left.
- For all the segmented lines found, the algorithm checks for potential overlap and proximity, erases those that are too similar and connects lines that follow upon each other. In detail:
    - The algorithm checks if points of two lines are closer than 1.5 pixel. If such a point exists, the point is deleted and if it is not located at the extremities of a given line, two new lines are generated from the initial line (the deleted point is in between these two lines).
    - Once proximities within a line to another are avoided, the algorithm allows for end to end joining of remaining lines. Lines are first listed for potential end joining with criteria that the maximal distance between two line extremities is set to 1 pixel. Once

all possible connections are listed, the algorithm chose the most likely based on maximal proximity and proceed to end joining.

- Finally, the algorithm filters the segmented lines for a minimal length of four pixels along the position axis.

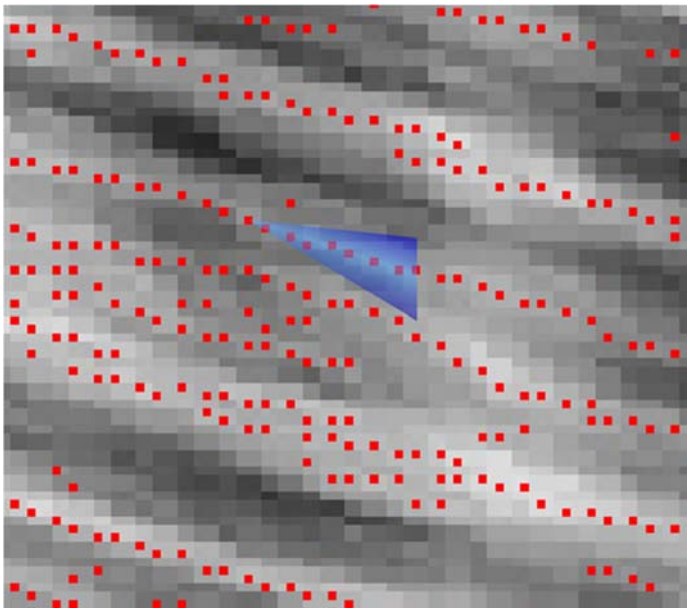

Figure S4. Search for trajectory. After the algorithm has found local intensity peaks in the kymograph (red dots), it links these peaks given the local average intensity; the search to link one peak to another is therefore limited to a window oriented toward the most probable velocity (blue triangle).

Determination of individual particle velocities

In a kymograph, the velocity of a moving particle can be readily deduced from the slope of the kymograph line belonging to the particle. In many applications of kymographs, only straight lines were fitted to kymograph lines, disregarding potential changes in velocity along the way. *KymographDirect* was explicitly designed to determine location-dependent velocities, requiring a more complex algorithm to accurately determine velocities. The application extracts the velocity of each individual particle by estimating the local slope of its trajectory. Our algorithm consists of the following steps:

- In case a trajectory contains less than 30 points, additional points are added by linear interpolation, adding one point in the middle of each pair neighboring points. This is repeated until the number of points is larger than 30.

  To avoid artifacts due to edge effects when fitting trajectories, points are added to both beginning and ending of each trajectory. These extra points are only used for fitting. For the beginning of a trajectory, the points are obtained by reflecting points 2 to 10 with respect to an origin of point reflection. This origin of point reflection is the time value obtained at the position of the first point from a linear fit to points 1 to 10 (Figure S5). The extra points at the end of the trajectory are obtained in the equivalent way. As a result, each trajectory is added with 18 points.
- Each trajectory is fitted over a sliding window of 19 points with a third-order polynomial. The local slope of the trajectory is determined by calculating the slope at the tenth (middle) point

of each fitted polynomial. The location-dependent velocity of the trajectory is obtained by sliding the 19-point window from one position to the next over the whole trajectory.

- The location-dependent velocity is converted to the proper units and scaling, and filtered with a first-order low-pass filter to reduce noise.

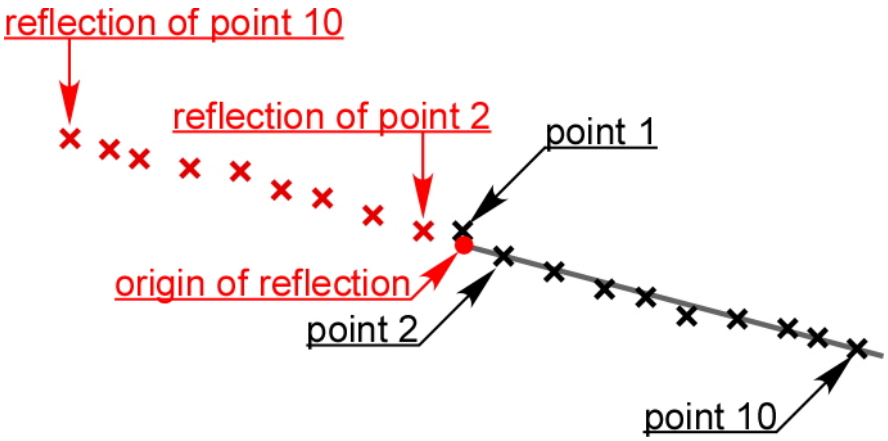

Figure S5. To avoid edge effects when fitting the trajectories, extra points are added to beginning and end of a trajectory for fitting purpose only. In the beginning of the trajectory reflections of points 2 to 10 are added. The reflection is performed with respect to the origin of reflection obtained from a linear fit to points 1 to 10 (grey line).

Determination of particle intensities

Another key property of particle trajectories is their location- or time-dependent intensity. In case the particles are only moving in one direction, a simple pixel-intensity summation around the particle position is sufficient to evaluate the fluorescence intensity. In the general case, however, different particles move in opposing directions, or are static. In this case, summation is not sufficient since it does not separate the signals caused by these different contributions. Use of the Fourier-filtered kymographs can be beneficial, since it allows for the separation of these contributions.

The information in the Fourier space is distributed in the following way: in the center of the space (at frequency 0) resides the average intensity of all signals (for a kymograph this represents the average of static and non-static signals); the (complex) intensity of other pixels represents the amplitude and phase of non-spatially uniform features of an image, and the position of the pixel corresponds to a specific spatial frequency of this image. When Fourier-filtering the image, as shown on Figure S1, what is obtained is the sum of individual contributions of the spatial frequencies selected (frequency 0 is filtered out). To further explain the effect of such filtering, we give an example on Figure S6A of a sinusoidal signal in one dimension; the signal s is given by $s = \cos(x) + 1$, where x is the position. This signal is positive. The Fourier transform displays a peak at frequency 0 (the signal average) and two peaks at frequency -1 and +1 (the oscillation; Figure S6B). When filtering out frequency 0 from this Fourier transform and applying the inverse Fourier transform, the signal obtained has the same amplitude as the original signal, but its average value is different and negative signal values appear.

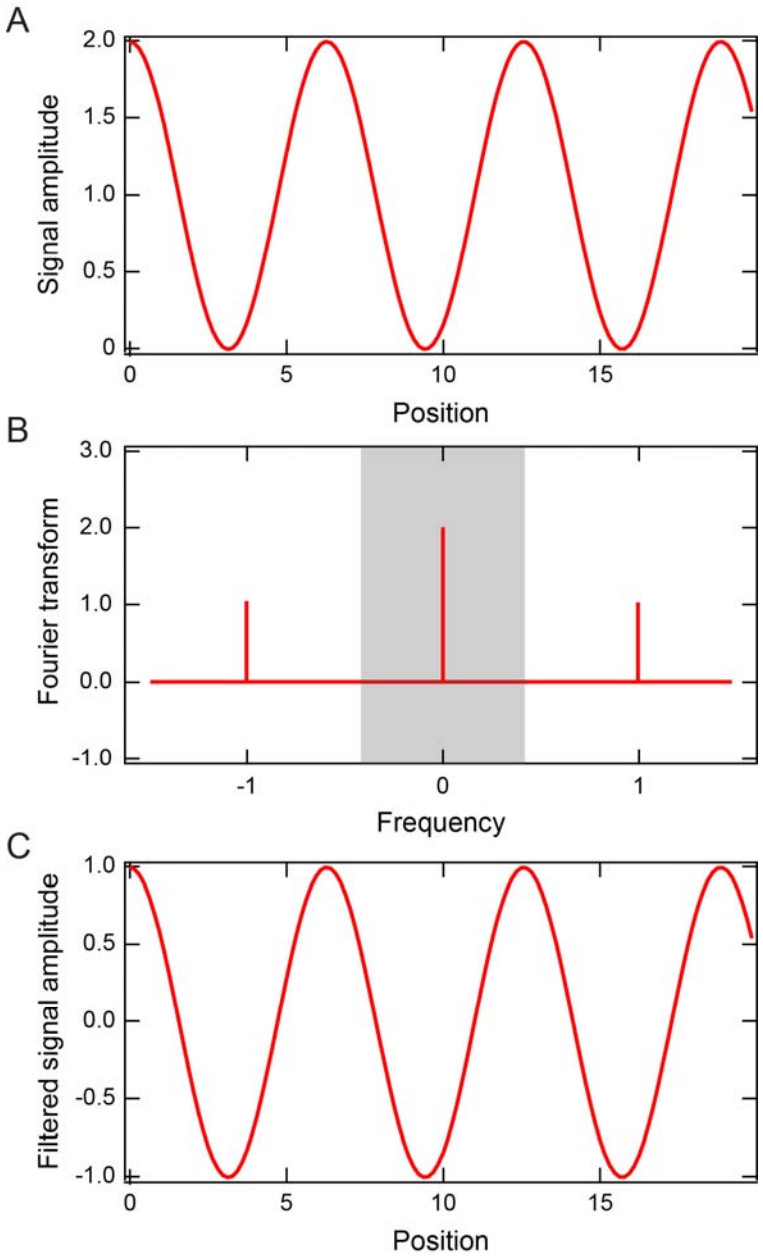

Figure S6. Effect of Fourier filtering on a one dimensional signal. (A) Original signal as a function of position. (B) Fourier transform of the signal (A). (C) Signal obtained after filtering its zeroth frequency in the Fourier domain.

Fourier filtering 2D data (as in the program) results in a similar effect. The amplitude of signals (trajectories in kymographs) is conserved, but the average of the signals is modified. In other words, the reference of the signal is lost. To correct for this, we use reference values in each kymograph, making use of pixels in the kymograph where there are no trajectories. To this end, the algorithm computes for each kymograph the average value of the 3% dimmest pixels and uses this value to set the zero intensity of the filtered kymograph. It is important to note that this can only work accurately when at least a small fraction of the Fourier-filtered kymograph pixels are devoid of particles.

After determining the correct intensity of the Fourier-filtered kymographs, the algorithm determines particles intensities by adding the intensities of pixels along the position axis centered on the particle

position. The number of pixels to be considered depends on the actual particle size, the resolution of the microscope and the particle velocity (due to blurring), parameters that are specific for each experiment. We use a default value of five pixels, but this can be modified by the user to fit specific experiment conditions.

Determination of particle mean square displacement

We use the common definition for the mean square displacement ρ_n of a particle for a time lag $n\Delta t$, where Δt is the time per frame:

$$\rho_n = \frac{1}{N-n}\sum_{i=1}^{N-n}(x_{i+n} - x_i)^2$$

where x_i is the position of the particle at time $i\Delta t$.

Linking trajectories

Once trajectories have been obtained using the different modes of analysis of *KymographDirect*, they can be linked, which is particularly useful in case the program extracts an apparently single trajectory as separate segments. The algorithm to link two trajectories works as follows:

- if two sub-trajectories are entirely separated in time (when the complete first trajectory occurred before the second), the extremities of the trajectory are linked. Note that no new time point is created in this process.
- if two trajectories do share several time points, the algorithm first finds the time point where trajectories are closest in position. Then the algorithm selects the time points within the trajectory occurring earliest in time, until (and including) the point of closest proximity. For the later trajectory, the algorithm selects the time points strictly after the time point of closest proximity.

The characteristics of the newly created trajectory, such as velocity and intensity, are obtained from the concatenation of the results of the analysis obtained for its sub-trajectories (with the data of non-retained time points omitted). The mean square displacement calculation as well as the run-length measurement are run on the newly created trajectory.

All results can be saved in separate files.

Output parameters of *KymographDirect*

KymographDirect gives a range of outputs, all representing important motility parameters:

- Particle position versus time of single trajectory
- Velocity versus time of single trajectory
- Intensity versus time of single trajectory
- Velocity versus position of single trajectory
- Intensity versus position of single trajectory
- Velocity versus position averaged over all trajectories
- Intensity versus position averaged over all trajectories
- Time-averaged velocity of single trajectory
- Histogram of time-averaged velocities of all trajectories

- Time-averaged intensity of single trajectory
- Histogram of time-averaged intensities of all trajectories
- Time-averaged track intensity versus position
- Run length
- Mean square displacement versus time lag

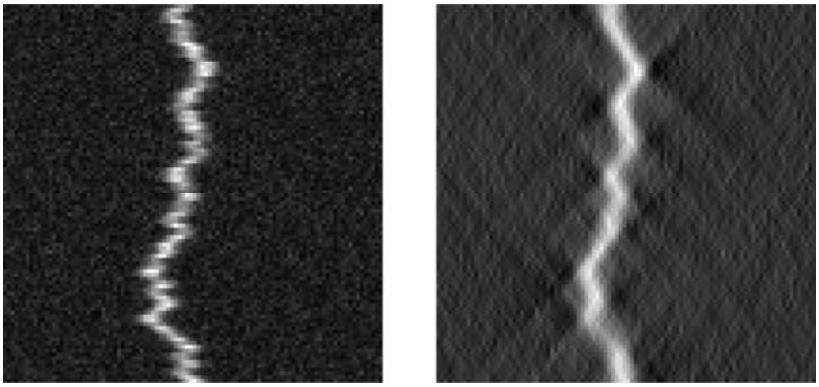

Figure S7. Diffusive particle: effect of Fourier filtering on the kymograph. Left, kymograph of a particle following a random walk, with D = 1 pixel²/frame and SNR = 6.8. Right, same kymograph Fourier filtered with the filter tailored for diffusive particles. It is noticeable that with Fourier filtering the most rapid changes of direction are smoothened.

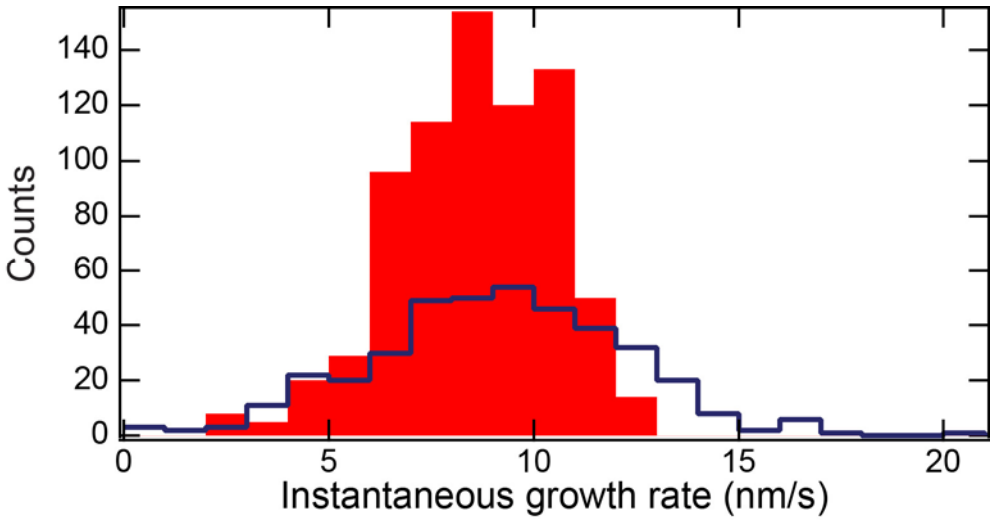

Figure S8. Comparison of *in vitro* microtubule growth rates obtained using *FIESTA* and *KymographDirect* using the same data set. Distribution of instantaneous growth rates obtained from 9 microtubules in the image sequence with *FIESTA* (blue) and *KymographDirect* (red).

Movie figure legend

Movie S1. Intraflagellar transport in *C. elegans* phasmid cilia. OSM-3 kinesin is fluorescently tagged with EGFP. Movie recorded at 6.6 frames per second and sped up twice for display. Scale bar, 2 µm.