**Supporting Appendix**

![Graph A](image1.png)

**A**
Surface-immobilized quantum dot

![Graph B](image2.png)

**B**
Quantum dot attached to a microtubule on top of kinesin motors

**Fig. 6.** Spatial accuracy of the QD tracking. (A) Histogram of the x-position (serving as an arbitrary axis) of a surface-immobilized QD emitting an average of 4,500 photons per frame (exposure time: 100 ms, no optovar). The continuous line shows a Gaussian fit with a width of 2.1 nm. This value is in good agreement with the theoretical prediction (see Methods). (B) To characterize the tracking accuracy of QDs bound to motile MTs, we used data from the two-motor case (as in Fig. 2E). We corrected for the MT movement by subtracting the fitted steps (from the step-finding algorithm) and plotted the histogram of the resulting positions. The continuous line shows a Gaussian fit with a width of 3.1 nm.
**Fig. 7.** Determination of the kinesin position around which the MT swivels. $x$-$y$-trajectory (red curve) of a QD bound to a swivelling MT in the one-motor case. The circular fit (blue line) was performed in a time interval where the QD displacement in radial direction was negligible. The position of the kinesin obtained with the fit is represented by the green cross.

**Supporting Text**

**Calculation of the Step Size of a Linear-Movement-Subtracted Staircase Generated by a Poisson Stepper.** Consider a Poisson stepper $x(t)$ that starts at $x = 0$ and takes steps of size $d$ at an average rate $k$. After time $T$, the stepper is at position $x(T)$. The mean speed of the stepper is $d/k$. Now the expected value of the position is:

$$E\{x(T)\} = dkT = vT.$$  

Thus, an estimator of the speed of a Poisson stepper is $v = \frac{x(T)}{T}$, being a better estimator than the slope of the linear regression (in the sense that it has less variance). The blue lines in Fig. 8 (in this appendix) correspond to the linearized movement and have slopes of $x(T)/T$. The expected value of the mean square value of the linear-movement-subtracted traces $y(t)$ (red curves) is:

$$E\{y^2(t)\} = \frac{1}{6}d^2kT = \frac{1}{6}dE\{x(T)\}$$ (see below for the detailed derivation). Replacing the expectation by the measured values gives an estimator of the step size:

$$d = 6\frac{\langle y^2(t)\rangle_T}{x(T)}.$$

**Definitions.**
Expectation. The expected value of \( x \) at time \( t \) is \( E\{x(t)\} = dkt \), where \( kt \) is the expected number of steps. The second moment of \( x(t) \) for a Poisson stepper is 
\[
E\{x^2(t)\} = d^2kt + (dkt)^2.
\]
The variance is then expressed by
\[
Var\{x(t)\} = E\{x^2(t)\} - (E\{x(t)\})^2 = d^2kt,
\]
being proportional to the expected number of steps \( kt \).

Mean value. The most useful experimental measurement of a time course \( f(t) \) is its mean value defined by
\[
\langle f(t) \rangle_T = \frac{1}{T} \int_0^T f(t) dt.
\]

Results

Estimator of the Velocity.
\[
E\left\{ \frac{x(T)}{T} \right\} = \frac{dkt}{T} = \nu.
\]
Note that
\[
Var\left\{ \frac{x(T)}{T} \right\} = \frac{d^2kT}{T^2} = \frac{\nu^2}{kT} = \frac{\nu^2}{N},
\]
where \( N \) is the expected number of steps. You cannot have an estimator that does any better than this for a Poisson process.

Mean Values.
\[
\langle y^2(t) \rangle_T = \left\langle \left( x(t) - \frac{t}{T} x(T) \right)^2 \right\rangle_T
\]
\[
= \left\langle x^2(t) - 2 \frac{t}{T} x(t) x(T) + \frac{t^2}{T^2} x^2(T) \right\rangle_T
\]
\[
= \langle x^2(t) \rangle_T - 2 \frac{x(T)}{T} \langle x(t) \rangle_T + \frac{x^2(T)}{T^2} \langle t^2 \rangle_T
\]

Special Result. (This is where we have to use Poisson statistics.)
\[
E \{ x(t)x(T) \} = \sum_{n=0}^{\infty} \sum_{m=0}^{\infty} n \Pr \{ x(t) = n \} m \Pr \{ x(T) = m \mid x(t) = n \} \\
= \sum_{n=0}^{\infty} \sum_{m=0}^{\infty} n \Pr \{ x(t) = n \} m \Pr \{ x(T-t) = m-n \} \\
= \sum_{n=0}^{\infty} \sum_{m=n}^{\infty} \frac{(kt)^n}{n!} e^{-kt} m \frac{(T-t)^{m-n}}{(m-n)!} e^{-k(T-t)} \\
= \sum_{n=0}^{\infty} \sum_{j=0}^{n} \frac{(kt)^j}{n!} e^{-kt} j! \frac{(T-t)^j}{j!} e^{-k(T-t)} \\
= \sum_{n=0}^{\infty} n(n+k(T-t)) \frac{(kt)^n}{n!} e^{-kt} \\
= k^2 t^2 + kt + kt \cdot k(T-t) \\
= kt + k^2 tT
\]

Note that this gives the right answer for \( t = T \).

**Expectations of the Mean Values.**

\[
E \{ \langle x(t) \rangle \} = \langle E \{ x(t) \} \rangle_T = \langle dkt \rangle_T = \frac{1}{\tau} dkT
\]

\[
E \{ \langle x^2(t) \rangle \} = \langle E \{ x^2(t) \} \rangle_T = \langle d^2 kt + d^2 k^2 t^2 \rangle_T = \frac{1}{\tau} d^2 kT + \frac{1}{\tau} d^2 k^2 T^2
\]

\[
E \{ \langle tx(t) \rangle \} = \langle E \{ tx(t) \} \rangle_T = \langle tE \{ x(t) \} \rangle_T = \langle dkt^2 \rangle_T = \frac{1}{\tau} dkT^2
\]

\[
E \{ \langle x(T)x(T) \rangle \} = \langle tE \{ x(T)x(T) \} \rangle_T = \langle td^2 k^2 T + td^2 kT \rangle_T = \frac{1}{\tau} d^2 T \left( k^2 T^2 + kT \right)
\]

(which follows from the special result above)

\[
E \{ \langle T^2 \rangle \} = \frac{1}{\tau} T^2
\]

**Derivation of the Main Result.**

\[
E \{ \langle y^2(t) \rangle \} = \frac{1}{\tau} d^2 kT + \frac{1}{\tau} d^2 k^2 T^2 - \frac{2}{T^2} \frac{d^2 \left( k^2 T^2 + kT \right)}{T^2} + \frac{d^2 \left( kT + k^2 T^2 \right)}{T^2} \frac{1}{T^2} T^2 \\
= \frac{1}{\tau} d^2 kT + \frac{1}{\tau} d^2 k^2 T^2 - \frac{2}{\tau} d^2 k^2 T^2 - \frac{2}{\tau} d^2 kT + \frac{1}{\tau} d^2 kT + \frac{1}{\tau} d^2 k^2 T^2 \\
= \frac{1}{\tau} d^2 kT \\
= \frac{1}{\tau} d \cdot E \{ x(T) \}
\]

Thus, we arrive at the main result, where we have used \( E \{ x(T) \} = dkT \).
Note that because the standard deviation of the step size $d$ is inversely proportional to $x(T)$, we can calculate a weighted average value of the step sizes calculated with different QDs using the following formula: 

$$
\bar{d} = \frac{\sum_i 6 < y^2 >_i}{\sum_i x_i(T)}.
$$
Fig. 8. Linearized-movement correction of a Poisson stepper. Examples are given for the one-motor case (A), the two-motor case (B), and the many-motor case (C). Black line: walked distance vs. time; red line, linear-movement corrected distance vs. time; blue line, linear movement with the slope of $x(T)/T$.

![Graphs A and B](image)

Fig. 9. Dependence of sideways motion on the number of kinesin motors. QDs were in the middle between two motors in the two- and three-motor case. (A, C, and E) $x$-$y$ trajectories of individual QDs bound to a MT for the two-motor case (A), the three-motor case (C), and the multimotor case (E). (B, D, and F) Sideways motion corresponding to A, C, and E, respectively. Note that the sideways motion decreases with the number of motors carrying the MT, as also previously observed in [Malik, F., Brillinger, D. & Vale, R. D. (1994) Proc Natl Acad Sci USA 91:4584-4588]. Because the distance between the kinesins involved in transport is the only parameter that changes, the decrease of the sideways motion indicates that the noise in the direction perpendicular to the MT direction is linked to the thermal fluctuations of the MT. Therefore, the extent of sideways motion is not determined by the limited accuracy of the detection system.
Fig. 10. Detection of MT buckling in the sideways motion. (A) Multicolor imaging of a MT gliding over three kinesin motors. The merged image shows the MT in red, the kinesin positions in green, and the QD position in blue. (B) x-y trajectory of the tracked QD. The asterisk indicates the beginning of the trajectory. (C) Projected walked distance (red curve) and sideways motion (green curve) of the QD positions along the pathway shown in B. The projection is made along the line determined by the motion of the QD at the beginning and the end of the trajectory, when three motors are involved in transport (blue background). The temporal period (yellow background) during which the MT buckles is clearly visible in the sideways motion where the QD position deviates in perpendicular direction up to 200 nm from its original pathway. We believe that the buckling is initiated by the release of the middle motor from the MT. In fact, before release, this motor might have been strongly stretched, because the onset of the buckling is associated with a distinct jump in the projected walked distance (see our arguments regarding the jumps in the three-motor case in the main article).
A 8 nm steps - no optovar- fixed Tetraspeck beads on the surface

B 8 nm steps - no optovar- QD on a MT

C 8 nm steps - optovar 1.6 - QD on a MT

D 4 nm steps - optovar 1.6 - QD on a MT

E Forward step sizes from (D) obtained by the step-finding algorithm
Fig. 11. Accuracy of the step size determination (longitudinal motion of QDs). In order to determine the minimum step size that our system was able to detect, we performed control experiments by applying artificial steps of different step sizes. QD-coated MTs were immobilized on a kinesin-coated surface in presence of AMP-PNP (nonhydrolysable form of ATP) and a square-shaped voltage was applied to a piezoelectric stage (Physik Instruments, Waldbraun, Germany) holding the sample. A period of 2 s with different voltage amplitudes (0.1 to 0.4 mV through an electronic “box” that divided precisely the voltage by 100) was applied, and streams were acquired in exactly the same conditions as described previously. (A) x-Position vs. time of a TetraSpeck bead bound to the surface. Eight-nanometer steps were applied every second. The imaging parameters were identical to those used for data acquisition on moving QDs (10 frame per second, 100-ms exposure, same optical power for fluorescence excitation). No opto var was used for this acquisition. (B–D) x-position vs. time of a QD bound to a MT attached to the surface by two to three kinesins. The motion of the MT was stopped by using the nonhydrolyzable ATP analogue AMP-PNP. Eight-nanometer steps were applied every second in B and C, where image acquisition was performed by using no opto var (B) or a ×1.6 magnifying opto var (C). Four-nanometer steps were applied every second in D, where a ×1.6 opto var was used for imaging. The global displacement of the QDs was due to stage drift that, in this case, could not be corrected because the TetraSpeck beads and the QDs underwent the same displacements. (E) Histogram of forward step sizes extracted by the step finding algorithm from the data of D. A double-Gaussian fit (continuous line, as in Fig. 3C) yielded a central value of $d = 4.2 \pm 0.1$ nm for the first peak. The distribution of step sizes has a width of $2.0 \pm 0.1$ nm and is thus very similar to the width of $1.8 \pm 0.2$ nm in Fig. 3C. We therefore believe that the width of step-size distribution in the two-motor case mainly originates from the noise of our detection system added to robust 4-nm steps and does not provide evidence for a spectrum of step sizes hidden in the distribution.

As seen in D and E, it is well possible to detect steps as small as 4 nm, demonstrating that the tracking accuracy of our imaging system was sufficient to detect such displacements in the cooperative stepping of kinesin motors.