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Supplementary Materials for

Direct observation of motor protein stepping in living cells using MINFLUX

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Materials and Methods

Sample preparation

DNA constructs

To generate pHTN-HaloTag-KIF5B, full-length human KIF5B (1-963) was cloned from a HeLa cDNA library and inserted into the pHTN-HaloTag expression vector (both a kind gift from the Ellenberg Lab, EMBL Heidelberg) by AQUA cloning (*29*). The pHTN-HaloTag-KIF5B[1-560] construct (HaloTag-K560) was generated equivalently, except using different primers to obtain the truncated KIF5B gene (1-560). To generate pHTN-HaloTag-MYO5B[K1701A], full-length human MYO5 was cloned from the same cDNA library as above and inserted into the pHTN-HaloTag expression vector using the NEBuilder HiFi DNA Assembly kit (New England Biolabs, Ipswich, MA, USA). Subsequently, the point mutation K1701A was introduced by AQUA cloning. The sequences of all constructs were verified by Sanger sequencing (Eurofins, Ebersberg, Germany) or next generation sequencing (Plasmidsaurus, Eugene, OR, USA). To generate the KIF5B[1-560]-HaloTag-FRB (K560-HaloTag) construct, the Halo tag was amplified from the pHalo-N1 backbone by PCR with additional restriction sites to allow for insertion into KIF5B[1-560]-GFP-FRB (*30*) after digestion to remove the GFP tag. Construct was verified by sequencing.

Protein purification

For motor-PAINT, *Dm*KHC (1-421)-SNAP-tag-6xHis (https://www.addgene.org/196975/) was purified from E. coli BL21 cells. Briefly, after transformation, bacteria were cultured until $OD_{600} \approx 0.7$ at 37°C. Cultures were cooled, after which protein expression was induced with 0.15 mM IPTG at 18°C overnight. Cells were then pelleted by centrifugation at 4500 × g, snap frozen in liquid nitrogen, and stored at −80°C until use. Cells were rapidly thawed at 37°C before being resuspended in chilled lysis buffer (50 mM sodium phosphate buffer supplemented with 5 mM $MgCl_2$, 5 mM imidazole, 10% [v/v] glycerol, 300 mM NaCl, 0.5 mM ATP, and 1 × EDTA-free cOmplete protease inhibitor; pH 8.0). Bacteria were lysed by sonication (5 rounds of 30 s), supplemented with 2 mg/mL lysozyme, and then incubated on ice for 45 min. The lysate was clarified by centrifuging at 26000 × g for 30 min before being incubated with equilibrated cOmplete His-tag purification resin for 2 hrs. Beads were then pelleted and resuspended in 5 column volumes (CV) wash buffer (50 mM sodium phosphate buffer supplemented with 5 mM MgCl2, 5 mM imidazole, 10% [v/v] glycerol, 300 mM NaCl, and 0.5 mM ATP; pH 8.0) four times. Finally, the resin was transferred to a BioRad column. Once settled, the wash buffer was allowed to elute before adding 3 CV elution buffer (50 mM sodium phosphate buffer supplemented with 5 mM $MgCl_2$, 300 mM imidazole, 10% glycerol, 300 mM NaCl, and 0.5 mM ATP; pH 8.0) to elute the protein. The eluent was collected, concentrated by spinning through a 3000 kDa MWCO filter, supplemented with 1 mM DTT, and 10% [w/v] sucrose before flash freezing in liquid nitrogen, and kept at −80°C. For labelling, protein was thawed and incubated with an additional 1 mM DTT for 30 min before adding 50 μM JF646-SNAP-tag ligand and incubating with rotation for 2 hours or overnight. Finally, protein was exchanged into wash buffer (low imidazole) supplemented with 2 mM DTT and 10% [w/v] sucrose by spinning through a 3000 Da MWCO filter. This also removes excess dye molecules as these are not retained by the filter. Concentration was determined with a BSA standard gel. All steps from lysis onwards were performed at 4°C.

Cell culture

U2OS NUP96-SNAP-tag cells (catalog no. 300444, CLS Cell Line Service, Eppelheim, Germany) (*31*) were cultured in DMEM growth medium with low glucose and without phenol red (Thermo Fisher Scientific, Waltham, MA, USA; Cat# 11880-028) supplemented with 1x MEM Non-essential amino acids (Thermo Fisher Scientific; Cat# 11140-035), 1x GlutaMax (Thermo Fisher Scientific; Cat# 35050-038), ZellShield (Minerva Biolabs, Berlin, Germany, Cat# 13-0050), and 10% [v/v] fetal calf serum (Thermo Fisher Scientific; Cat# 10270-106). Cells were grown in an incubator at 37°C, 5% CO2, and 100% humidity. High-precision 24 mm round glass coverslips (No. 1.5H; Marienfeld, Lauda-Königshofen, Germany; Cat# 117640) were cleaned by overnight incubation in methanol:hydrochlorid acid (50:50) stirring continuously, followed by subsequent washes with water until a neutral pH was achieved. Cleaned coverslips were then dried overnight under a laminar flow bench and cleaning was finalized by exposure to ultraviolet-radiation for 30 min. To utilize the sample stabilization system on the MINFLUX microscope, the prepared coverslips were coated with 0.01% poly-l-lysine (Sigma-Aldrich St. Louis, MO, USA; Cat# P4707) by applying 200 µL to each coverslip, incubating for 15 min, removing the solution, then applying 200 µL 200 nm gold nanoparticle solution (Nanopartz, Loveland, CO, U.S.A.) and incubating for 15 minutes under a running cell culture hood. Afterwards, the coverslips were rinsed twice with PBS and coating was finalized by UV radiation for 30 min. For imaging, 100k cells were seeded 3 days in advance on cleaned coverslips in a 6-well plate, each well containing 2 mL cell culture medium, to reach 50-70% confluency until the day of imaging. All experiments were performed with cells of different passage numbers.

Mouse primary cortex culture

Primary mixed sex cortical neuron cultures were prepared from prenatal embryos of CD-1® IGS Mouse embryos (Charles River; Strain Code: 022; RRID: IMSR_CRL:022) with no genetic modification at embryonic day 15 (E15). Animals were kept under standard SPF (specific pathogen free) conditions and sacrificed following routine and standard operating procedures of animal welfare with approval from the institutional animal care and use committee at EMBL. The embryonic cortex was dissected and collected in ice cold HBSS (containing Ca^{2+}/Mg^{2+}; Thermo Fisher Scientific; Cat# 14025092) from up to 15 mouse embryos. The tissue was washed thrice with ice cold HBSS (without Ca^{2+}/Mg^{2+}; Thermo Fisher Scientific; Cat# 14170112) and incubated with 0.25% Trypsin (Thermo Fisher Scientific; Cat# 15090046) in a water bath (37 °C) for 15 min. Afterwards, the tissue was washed again thrice with ice cold HBSS (without Ca^{2+}/Mg^{2+}) before dissociation/trituration in plating medium (Neurobasal; Thermo Fisher Scientific; Cat#: 12348017) containing 2% [v/v] B27 (Thermo Fisher Scientific; Cat#: 12587010), 1% [v/v] N2 (Thermo Fisher Scientific; Cat#: 17502048), 2% [v/v] GlutaMAX (Thermo Fisher Scientific; Cat#: 35050061), 1% [v/v] Pen/Strep (Thermo Fisher Scientific; Cat#: 15140122), 0.1 M NaPyr (Thermo Fisher Scientific; Cat#: 11360039), 10% [v/v] FBS (Thermo Fisher Scientific; Cat# 10270106), and 1 mg/mL DNAse I (Sigma Aldrich/Merck; Cat# 11284932001). The cell solution was passed through a 70 µm strainer and counted using Cellometer Auto T4 (Nexcelom Bioscience, Lawrence, MA, USA). 400,000 cells were plated on 24 mm coverslips pre-coated with 0.1 mg/mL Poly-D-Lysine (Sigma Aldrich; Cat# P0899), 2.5 µg/mL laminin (Sigma Aldrich; Cat#: 11243217001) and the gold beads. After 1 hour, a full medium change was performed using culturing medium (Neurobasal containing 2% [v/v] B27, 1% [v/v] N2, 2% [v/v] GlutaMAX, 1% [v/v] Pen/Strep, 0.1 M NaPyr). Cells were maintained at 37 °C in a humidified incubator with an atmosphere of 95% air and 5% CO_2 until days in vitro (DIV) 8-10. A ⅓ to ½ medium change was performed twice a week.

Motor-PAINT

For motor-PAINT, U2OS cells or neurons were permeabilized for 1 minute in extraction buffer (BRB80: 80 mM K-PIPES, 1 mM MgCl2, 1 mM EGTA; pH 6.8, supplemented with 1M sucrose and 0.15% [v/v] TritonX-100) pre-warmed to 37°C. Pre-warmed fixation buffer (BRB80 supplemented with 2% [w/v] formaldehyde (FA) was added to this (i.e. final FA concentration of 1% [w/v]) and the solutions were mixed by gentle pipetting for 1 min. The sample for Fig. 1G track 2 & 3 was treated with 0.015 % of glutaraldehyde instead of FA. Subsequently the extraction-fixation-solution was exchanged for pre-warmed wash buffer (BRB80 supplemented with 1 µM Taxol) and incubated for 1 min. The 1-minute wash step was repeated three more times. Subsequently, staining solution (1 to 1000 dilution, BioTracker 488 Live Cell Microtubule Dye, Sigma-Aldrich, in BRB80) was added and incubated for at least 1 hr to stain the microtubules in case cells were not transfected for GFP-α-tubulin (Fig. 1D, G track 2 & 3, 3C, D). This solution was removed, and the chamber was washed once for 1 min with wash buffer (BRB80 supplemented with 1 µM Taxol). The wash buffer was then exchanged for pre-warmed imaging buffer (BRB80 supplemented with 583 µg/mL catalase, 42 µg/mL glucose oxidase, 1.7% [w/v] glucose, 1 mM DTT, 2 mM methyl viologen, 2 mM ascorbic acid, 1 µM Taxol, and 5 mM or 10 µM ATP for high ATP or low ATP preparations, respectively) containing ≈ 0.5 nM kinesin.

Transfection of U2OS cells

One day after seeding, cells were transfected using lipofectamine 2000 (Thermo Fisher Scientific; Cat# 11668019). For each coverslip, 3 µL lipofectamine together with motor protein plasmid DNA (50 ng/coverslip), GFP-α-tubulin (100 ng/coverslip) (*32*), and salmon sperm DNA (850 ng/coverslip) were added to 100 µL Opti-MEM Reduced Serum Medium with GlutaMAX Supplement (Thermo Fisher Scientific; Cat# 51985026) and mixed well by vortexing. After a 10 min incubation, the cell culture medium was exchanged for 2 mL Opti-MEM and 100 µL of the transfection solution was added dropwise per coverslip. Cells were cultured in an incubator at 37 °C and 5 % CO_2 overnight and the transfection medium was exchanged for cell culture medium the next day. Kinesin was co-transfected with GFP-α-tubulin (*32*) while MYO5 was co-transfected with GFP-Lifeact.

Transfection of mouse primary neurons

Primary mouse neurons were transfected at DIV2 or 3 using calcium phosphate precipitation. For one coverslip (Ø 24 mm), sterile double distilled H_2O, (added up to 60 µL) was mixed with 6 µL of 2 M $CaCl_2$, and 6 µg total endotoxin free plasmid DNA (4 µg HaloTag-KIF5B[1-560] plasmid DNA + 2 µg of GFP-α-tubulin) isolated using the NucleoBond Xtra Midi Plus EF kit (MACHEREY-NAGEL GmbH & Co. KG, Düren, Germany; Cat# 740422.50). The mixture was incubated for 5 min at room temperature (RT). Next, 60 µL of 2× HBS buffer (274 mM NaCl, 9.5 mM KCl, 1.4 mM Na_2HPO_4, 15 mM HEPES, 15 mM glucose, pH 7.14) was added followed by vortexing briefly. The mixture was then incubated for 25 min at RT. Prior to transfection, conditioned culturing medium was removed from the cells (and stored) and replaced with Neurobasal (Thermo Fisher Scientific; Cat# 12348017) stored in the incubator at 37°C overnight. Subsequently, 120 µL of the transfection mixture was applied dropwise per coverslip followed by an incubation of 30-40 min at 37°C in the incubator until the precipitate was formed. The cells were washed two times with 1× HBS (pH 6.9) for 5 min in the incubator and finally once with Neurobasal (kept in the incubator overnight) for 5 min in the incubator. After the Neurobasal wash,

the medium was replaced with the original conditioned culturing medium and cells were cultured until DIV8-10 for the live cell imaging experiment.

Live cell staining and microtubule stabilization

For live imaging of transfected cells, HaloTag live labeling of recombinant kinesin was performed with the JF646-HaloTag ligand (Janelia, Ashburn, VA, USA) on the morning of imaging. Cell culture medium was exchanged for 0.5 nM JF646-HaloTag ligand in PBS and incubated for 15 min at 37 °C and 5 % CO_2 before changing back to culture medium. For experiments with Taxol for stabilization of microtubules, the coverslip of interest was incubated with 10 μM paclitaxel (Taxol, Sigma-Aldrich) one hour prior to imaging, by addition to the cell culture medium and the cells were kept in the same media throughout imaging. For the experiment using K560-HaloTag, JFX646-HaloTag ligand (Janelia, Ashburn, VA, USA) was used.

Test sample preparation

For optimizing 2D tracking parameters, single Atto647N nanobody (20A_Atto647N, Biomers.net, Germany) deposited on a glass coverslip was used. We immobilized the dye by the following protocol. First, the coverslip surface was plasma-cleaned (Plasma Prep2; PIE Scientific, U.S.A.) for 5 min. Then we applied a solution of 2% APTES (Sigma-Aldrich, U.S.A.), 5% of water and 93% of Ethanol, incubated for 15 min at RT, washed twice with 100 % ethanol, washed twice with DI water, and placed the sample at 65 °C for 1.5 hrs. Then, a dye solution in DI water (1:100,000 dilution) was applied and the coverslip was incubated for 20 min at RT. After the incubation, the coverslip was washed with 100 mM NH_4Cl three times, washed with DI water for three times, and was dried overnight. For optimizing 3D tracking, fluorescent beads (50 nm TetraSpeck microspheres fluorescent blue/green/orange/dark red, C47281, Thermofisher, U.S.A.) were used. The beads were immobilized on a glass coverslip by incubating in 100 μM MgCl2 in water for 10 min and then the coverslip was washed twice with PBS.

Sample mounting

Samples were mounted on cavity slides (Brand GmbH & Co KG, Wertheim, Germany, Cat# 475505) containing approximately 150 μL imaging buffer for motor-PAINT, cell culture medium containing 20 mM HEPES buffer (pH 7.25) for live cells, air for the 2D tracking test sample, and PBS for the 3D tracking test sample. Edges of the coverslip were sealed with dental addition-curing duplicating silicone (Picodent twinsil; Dental-Produktions- und Vertriebs-GmbH, Wipperfürth, Germany) and cured for 5 min at 37 °C.

Microscopy

MINFLUX microscopy

A commercial MINFLUX microscope, Abberior MINFLUX (Abberior Instruments, Göttingen, Germany)(*14*), was used for all MINFLUX tracking experiments. In detail, we used a 100x oil immersion objective lens (UPL SAPO100XO/1.4, Olympus, Tokyo, Japan), 642 nm CW excitation laser, two avalanche photodiodes (SPCM-AQRH-13, Excelitas Technologies, Mississauga, Canada) with a detection range of 650 – 685 nm for the first detector and 685 – 760 nm for the second (detected photons were summed), and a pinhole size corresponding to 0.78 airy units. All the hardware was controlled by Abberior Imspector software (version 16.3.13924-m2112). The drift during the motor-PAINT measurements was minimized using the built-in stabilization system of the Abberior MINFLUX with typical drifts under 1 nm in xyz directions.

For the sample stabilization, 200 nm gold nanoparticles (Nanopartz, Cat# A11-200-CIT-DIH-1-10) deposited on the coverslip surface were used as a positional reference.

Tracking parameters and sequences

We applied an iterative localization approach (*11*) with decreasing L size, a different number of collected photons, and a different dwell time in each iteration, as listed in the tables below. During the last iteration, the system continued localizing a molecule with the same parameters until its abort criteria (either losing the detection signal or exceeding the predefined central frequency ratio (CFR) value) was met. The CFR is a ratio of detection frequencies at the center and at the offset (position 7 vs other positions, Fig. 1A), and was introduced in previous work (*14*) to reject biased localizations due to multiple emitters close to the targeted coordinate patter (TCP).

The following two tables show the parameters used for 2D and 3D tracking, respectively.

2D tracking	**1st iteration**	**2nd iteration**	**3rd iteration**	**4th iteration**
L size (nm)	284	302	151	75
TCP	Hexagon	Hexagon	Hexagon	Hexagon
Minimum number of collected photons (counts)	40	20	10	100**
Laser power multiplier *	1 ×	1 ×	2 ×	2.5 ×**
Minimum TCP dwell time (µs)	50	50	50	300
Pattern repeat	1	1	1	3
CFR	-1.0	0.5	-1.0	0.9
Background threshold (kHz)	15	30	30	40

* This is a multiplier on the reference laser power to achieve an actual power used in each iteration.
** In experiments for fig. S7, 180 photons were collected with the laser power multiplier of 3.5×.

3D tracking	**1st iteration**	**2nd iteration**	**3rd iteration**	**4th iteration**	**5th iteration**
L size (nm)	288	1440	288	151	100
TCP	Hexagon	Zline**	Octahedron	Octahedron	Octahedron
Minimum number of collected photon (counts)	40	30	40	40	150
Laser power multiplier*	1 ×	1 ×	1 ×	2.5 ×	6.0 ×
Minimum TCP dwell time (µs)	200	200	200	200	300
Pattern repeat	1	1	1	1	1
CFR	-1	- 1	0.5	- 1	1.0
Background threshold (kHz) * **	30	30	35	35	50

* A multiplier on the reference laser power to achieve an actual power used in each iteration.
** A TCP for an axial scan with two points above and below the focal plane.

*** In 3D tracking, an automatic background estimation feature was enabled. The background threshold value for the no-signal abort criterion was the sum of the automatically estimated value and the value listed in the table.

Reference laser power values for each experiment, measured at the back aperture of the objective lens, are listed in the table below.

	Fig. 1D to F	**Fig. 1 G to L**	**Fig. 2 A to M**	**Fig. 3**
Reference laser power (µW)	37	110	110	37

Imaging for parameter optimizations

For determining the tracking parameters, we first optimized a background threshold value in the following way. With the parameters listed in the table, we switched off the automatic background estimation function and set the background threshold to zero so that the system tracked the background at the last iteration. 5 kHz was added to the median value of the measured signals and was used as a threshold value for 2D tracking. By doing the same but with the automatic background estimation function on, we added 40 kHz for the 3D tracking. Then, we optimized the laser power (i.e. laser power factor) by tracking immobilized single Atto647N molecules and finding the power which gave a ≈150 kHz detection frequency. In addition, we tested different numbers of pattern repeats with a triangular and hexagonal TCP. Based on the overhead time for the pattern repeats, we set the repetition to 1 repeat per 100 µs, so it does not slow down our target temporal resolution (1 kHz localization rate) significantly but achieves sufficient averaging to counteract system instability. Finally, we optimized the number of photons to reach a ≈ 2 nm localization precision, which was over 100 photons per localization. For 3D tracking, we followed the same procedure but with a 40 nm fluorescent nanosphere in water instead of air.

Widefield microscopy

For a sample quality check to confirm kinesin motility in both motor-PAINT and live cells (movie S1 for motor-PAINT, S5 and S8 for HaloTag-kinesin-1 (full-length) and HaloTag-K560, respectively), we used a custom-built standard fluorescence microscope. The microscope details were described previously (*33*). Briefly, we used a 100× silicon oil immersion objective lens (UPlanSApo 100×/1.35 Silicon oil objective lens, Olympus, Japan), 638 nm excitation laser at 1.9 mW (iBeamMLE; Toptica, Munich, Germany), detection wavelength of 685/70 nm, and imaged on a sCMOS camera (Orca-Flash 4.0 V2, Hamamatsu Photonics, Japan) with a projected pixel size of 123 nm and an exposure time of 30 ms. The microscope was controlled by MicroManager 2.0.0 (*34*) with htSMLM (*35*) for image acquisitions.

Data processing and analysis

All MINFLUX data was processed, analyzed, and rendered on a custom MATLAB software, SMAP publicly available at GitHub (https://github.com/jries/SMAP)(*36*). We first determined the angle of individual tracks from the eigenvector of the covariance matrix of the positions. For displaying motor-PAINT data (Figs. 1D,E, 3C), we binned the position in 4 nm steps along the microtubule direction. For displaying localization data as super-resolution images, we rendered every localization as a Gaussian function with $\sigma = 3$ nm (Fig. 2B,F).

For stepping analysis, tracks were manually segmented, excluding diffusive tracks and tracks without clear steps. We also excluded static tracks and out-of-focus tracks with low detection

signal frequencies. Additionally, we manually excluded parts at the beginning or the end of the tracks without clear steps. We determined the time when a step occurred with a previously published step finding algorithm (*37*). Optionally, we merged two consecutive small steps (<5.5 nm for motor-PAINT and <11 nm for live-cell tracking) and split large steps (>11 nm for motor-PAINT and >22 nm for live-cell tracking). We validated the automated step finding by visual inspection. In rare cases, we manually corrected step positions when the algorithm failed to correctly identify steps. For further analysis we calculated the average position of each step. The offset distances of zigzag tracks were measured by taking the differences of coordinates of two consecutive steps in y-direction, which is the perpendicular axis to the kinesin walking direction.

For a statistical analysis of the found step sizes, we used Gaussian function fitting. To analyze distributions of dwell times t, we assumed that each 8 nm step is the result of two consecutive stochastic processes with kinetic constants k_1 and k_2. The two time-limiting steps correspond to a phosphate release followed by an ATP binding and an ADP release followed by an ATP hydrolysis (*17*). This interpretation is supported by our observation that a single exponential did not fit the initial part of the histogram. As a 16 nm step is composed of two 8 nm steps, we describe it by four consecutive stochastic processes with two distinct kinetic constants. These stochastic processes are described by hypoexponential distributions, i.e., the convolution of exponential distributions:

$$h(t, k_i) = \sum_{i=1}^{k} k_i e^{-k_i t} \left(\prod_{j=1, j\neq i}^{k} \frac{k_j}{k_j - k_i} \right).$$

For 8 nm steps $k_i = \{k_1, k_2\}$ and for 16 nm steps $k_i = \{k_1, k_2, k_1 + \epsilon, k_2 + \epsilon\}$ with $\epsilon \ll k_i$ to avoid numerical divergence. A fit of these distributions to dwell time histograms allowed us to extract k_1 and k_2. Average dwell times $\langle t \rangle$ are calculated from the fitted distributions to be independent of long stall times as:

$$\langle t \rangle = \sum t\, h(t) / \sum h(t)$$

We estimated the experimental localization precision σ for each track or step by calculating the standard deviation (SD) of coordinate difference between consecutive localizations:

$$\sigma_x = \frac{1}{\sqrt{2}} \sqrt{\frac{1}{N-1} \sum_{i=1}^{N-1} (x_{i+1} - x_i - \mu)^2} \quad \text{with} \quad \mu = x_N - x_1.$$

For static localizations with only random, uncorrelated noise, the variance of the coordinate differences is twofold higher than that of the coordinates themselves, leading to a $\sqrt{2}$ higher σ when calculated on the differences compared to when calculated on the coordinates directly. For mobile fluorophores, this equation over-estimates the noise. Correlated noise (e.g., drifts) on the other hand is underestimated.

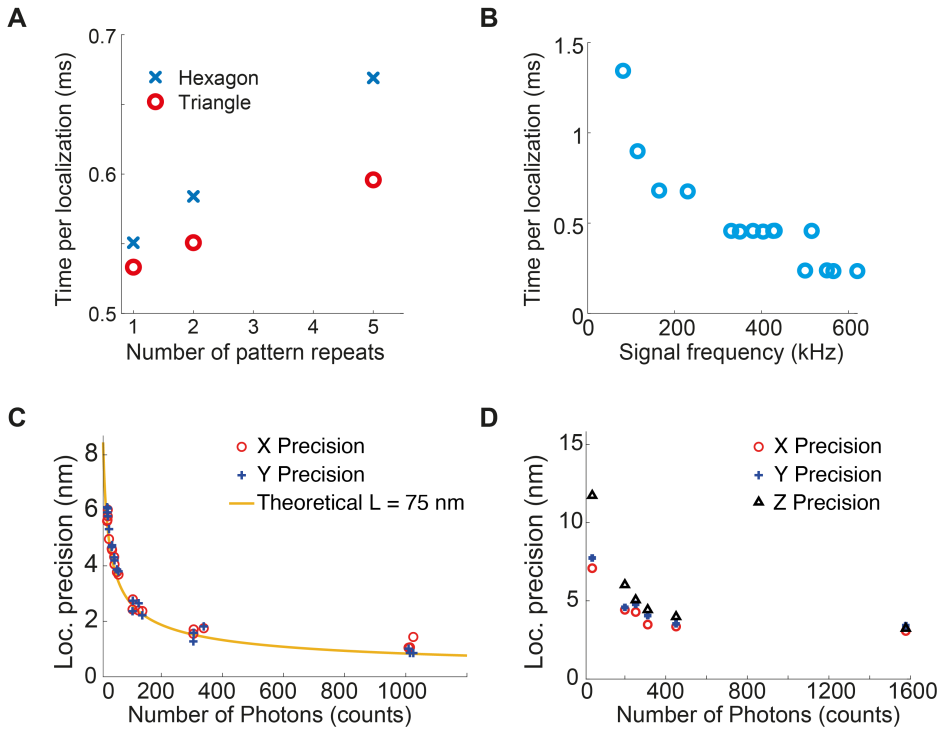

Fig. S1: Tracking parameter optimizations. (**A - C**) **2D tracking parameter optimization**. (**A**) Single molecules of Atto647N deposited on a glass coverslip were tracked with two (hexagonal and triangular) targeted coordinate patterns (TCP) and different numbers of pattern repeats, and the temporal resolutions per localization are compared. Although the hexagonal TCP takes more time, the difference is not decisive, as our target temporal resolution is 1 ms. Due to the higher accuracy of the localization, we selected the hexagonal TCP in all our 2D tracking experiments (*14*). (**B**) Single molecules of Atto647N deposited on a glass coverslip were tracked at different laser powers (and thus detection signal frequency) and the temporal resolutions per localization are compared. (**C**) Single molecules of Atto647N on a coverslip were tracked with different numbers of collected photons at the last iteration and the resulting experimental localization precisions (the standard deviation of coordinate differences between consecutive localizations) are plotted. (**D**) 50 nm fluorescent nanospheres deposited on a glass coverslip were tracked in 3D and the resulting localization precisions are plotted. See Materials and Methods for the tracking parameters used in our experiments.

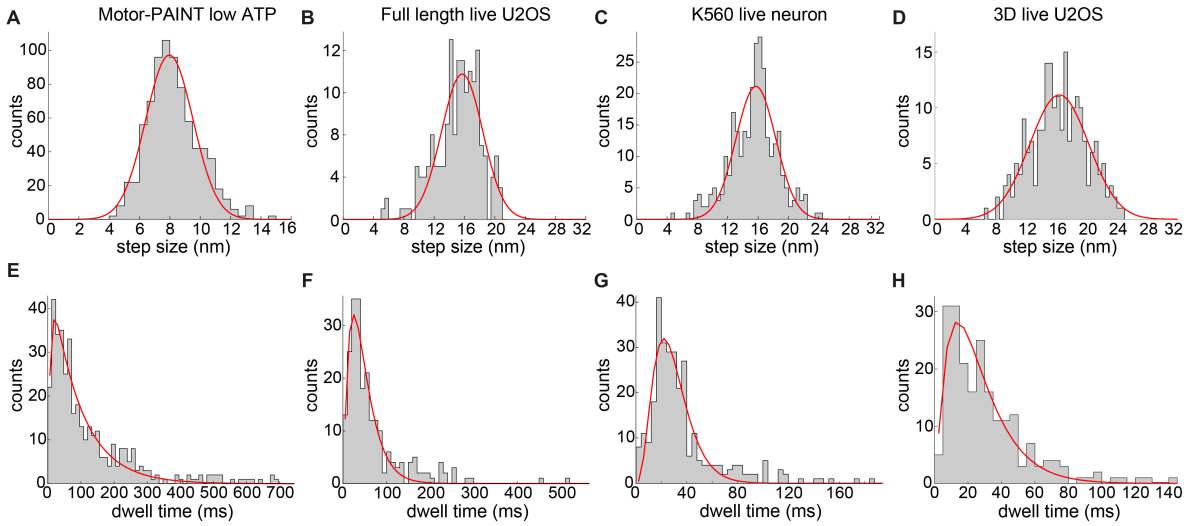

Fig. S2: Step size and dwell time histograms. (**A**, **E**) Step size and dwell time histograms from DmKHC (1-421)-SNAP-tag-6xHis tracking in motor-PAINT at low ATP concentration (10 µM) with a fitted step size of 8.0 ± 2.2 SD ± 0.11 SEM nm and an average dwell time of 97.1 ms (data from 5 experiments, 45 tracks, and 393 steps), (**B**, **F**) full-length wild type kinesin-1 in live U2OS cells with a fitted step size of 15.7 ± 3.8 SD ± 0.25 SEM nm and an average dwell time of 46.8 ms (data from 6 experiments, 10 tracks, and 234 steps), (**C**, **G**) HaloTag-K560 in live primary neurons with a fitted step size of 15.7 ± 3.7 SD ± 0.21 SEM nm and an average dwell time of 29.2 ms (data from 10 experiments, 23 tracks, and 303 steps), and (**D**, **H**) 3D tracking of HaloTag-K560 at mostly horizontal microtubules in live U2OS cells with a fitted step size of 16.4 ± 5.5 SD ± 0.37 SEM nm and an average dwell time of 26.3 ms (data from 18 experiments, 26 tracks, and 216 steps). Note that inclined trajectories are not included to avoid projection artefacts.

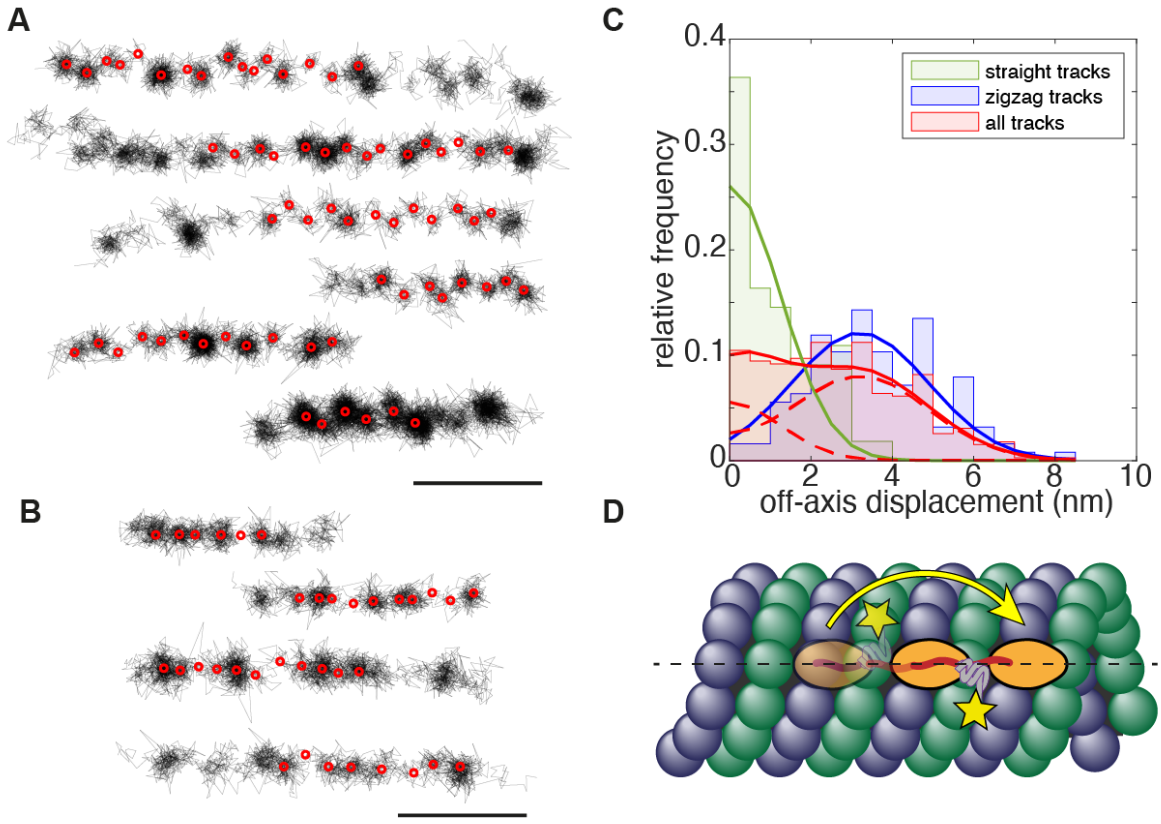

Fig. S3: Zigzag trajectories. (**A**) Example trajectories with clear zigzag offsets. The detected steps are marked with red circles. (**B**) Example trajectories without clear zigzag offsets. (**C**) Histograms of the off-axis displacement from the microtubule axis. Manually selected straight tracks (green, 55 steps in 8 tracks with the offset values of 1.1 ± 0.98 SD ± 0.13 SEM nm) were fitted with a Gaussian centered on zero and zigzag tracks (blue, 126 steps in 15 tracks with the offset values of 3.6 ± 1.6 SD ± 0.21 SEM nm) were fitted with a free Gaussian. All tracks (red, 393 steps in 45 tracks) were fitted with a combination of both distributions (dashed lines), indicating that 73% of the steps displayed zig-zag motion. (**D**) Schematic of a C-terminally labelled kinesin. When the label position is asymmetric relative to the connection of the motor domains, this will cause positional offsets perpendicular to the walking direction at each step. Scale bars: 50 nm.

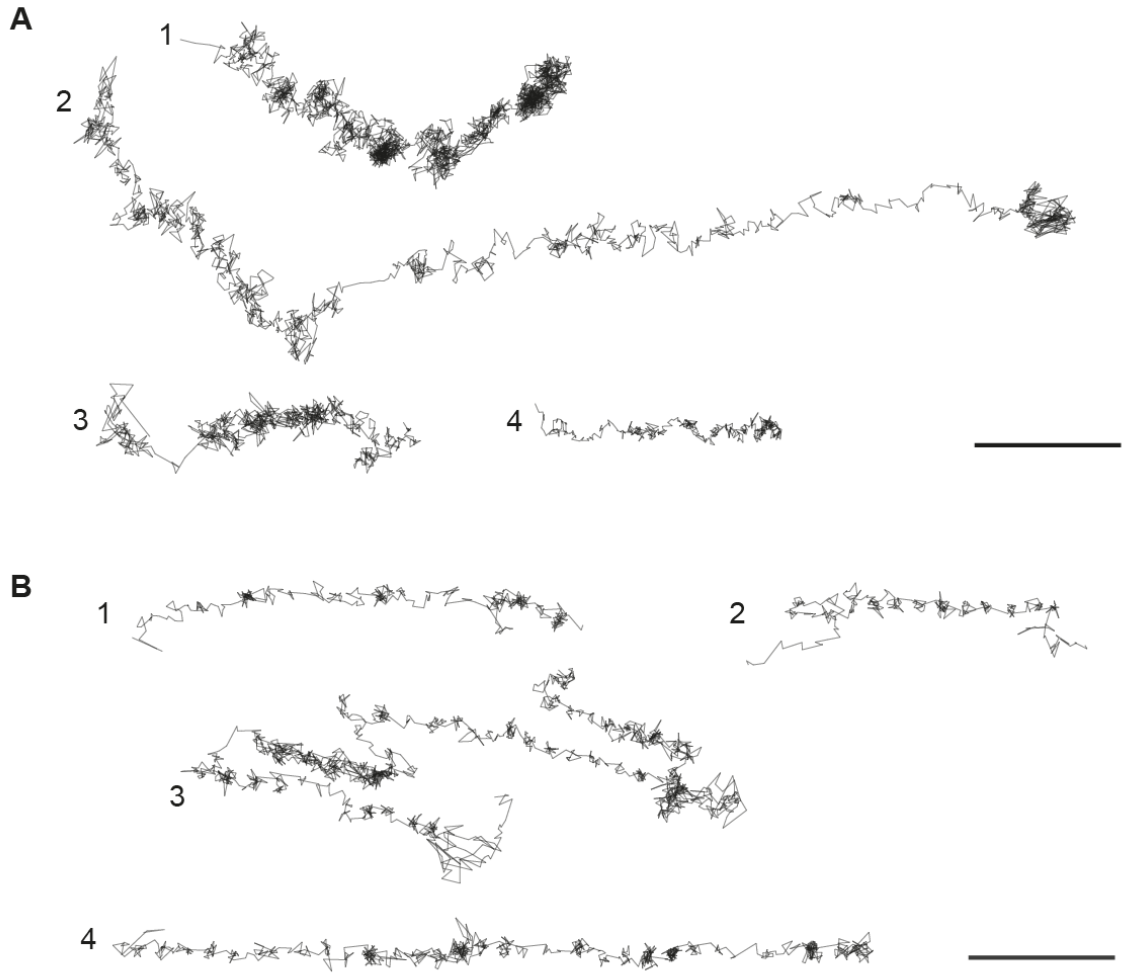

Fig. S4: Example trajectories of full-length wild type kinesin-1. (A) Trajectories without clear steps (9 tracks out of 19 tracks), indicating passive kinesin transport by attached cargos or mobile microtubules. (B) Trajectories with clear steps (10 out of 19 tracks) indicating active kinesin stepping/transport. Scale bars: 100 nm.

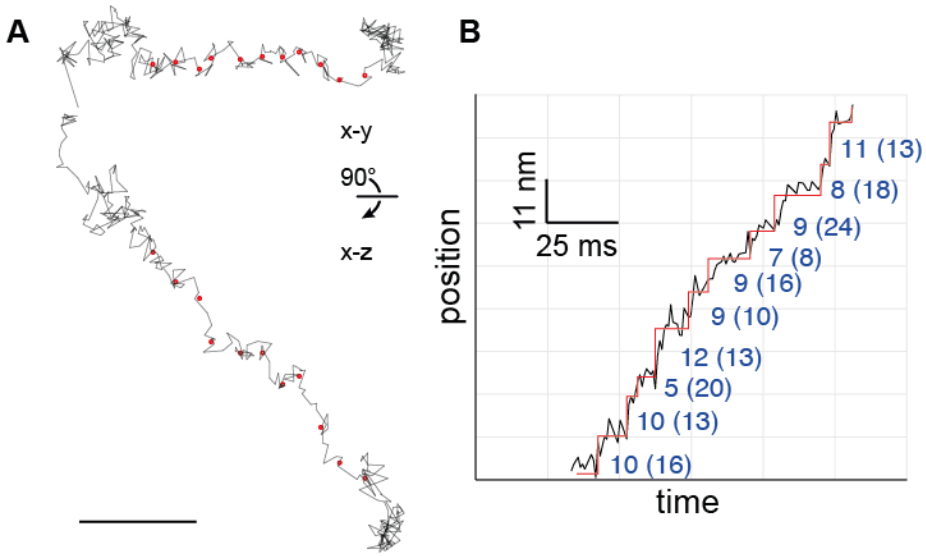

Fig. S5: 3D tracking avoids a projection bias of step sizes in inclined tracks. (**A**) A trajectory of HaloTag-K560 at an angle of 47 degrees in the xz plane captured by 3D MINFLUX tracking in a live U2OS cell, showing multiple steps (red circles). (**B**) Time versus position plot in a 2D analysis showed a bias towards smaller step sizes (9.0 ± 2.0 SD nm), compared with the corresponding steps in 3D analysis (shown in brackets, 15.1 ± 4.8 SD nm). Scale bar: 50 nm.

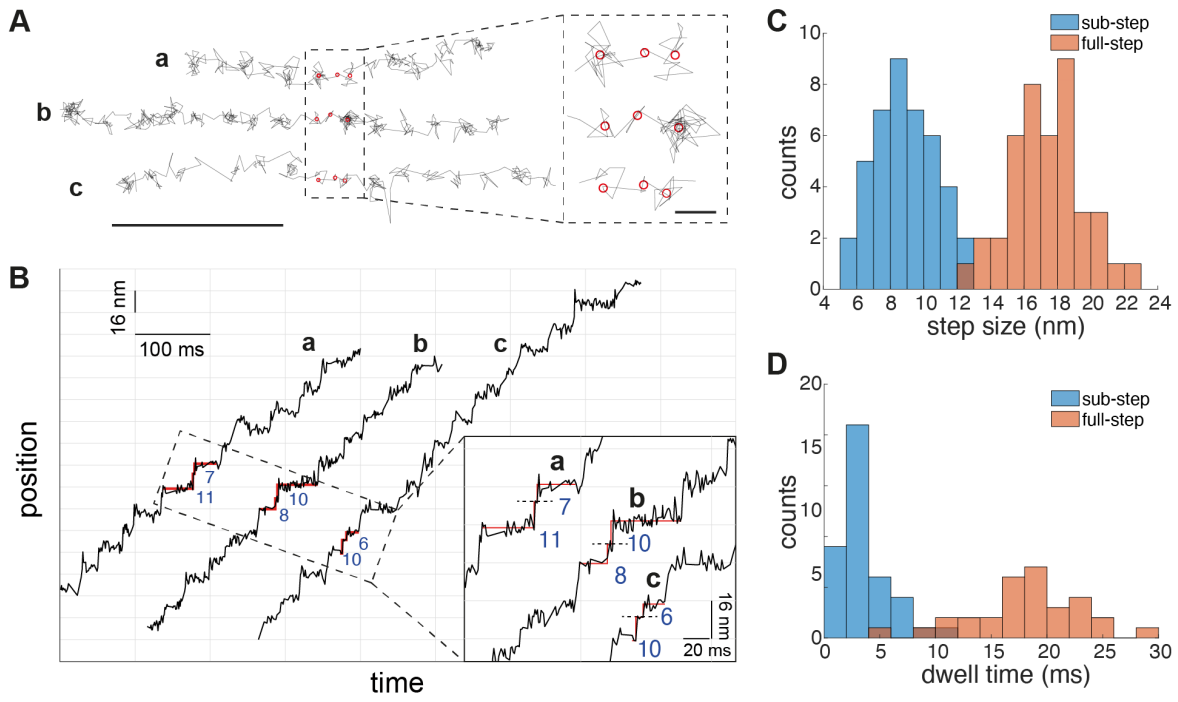

Fig. S6: Sub-steps of N-terminally labelled kinesin-1. A small fraction of steps (42 steps out of 2887 steps) showed sub-steps within 16 nm full-steps. (**A**) Representative tracks of N-terminally labelled HaloTag-K560 in live U2OS cells as line plots connecting each localization. Three consecutive steps (including a sub-step) are marked with red circles. (**B**) Corresponding time versus position plots (black line) and the fitted curve (red line) at the sub-steps (horizontal dotted lines). (**C**) A histogram of the fitted sub-steps (8.9 ± 1.8 SD ± 0.28 SEM nm) and full-steps (17.4 ± 2.2 SD ± 0.33 SEM nm). (**D**) A histogram of the dwell time with a mean value of 3.7 ms and 25 ms for sub-steps and full-steps, respectively. Data for (C, D) are from 33 experiments, 42 tracks, and 42 steps. Scale bar: 10 nm (A inset), 100 nm (A).

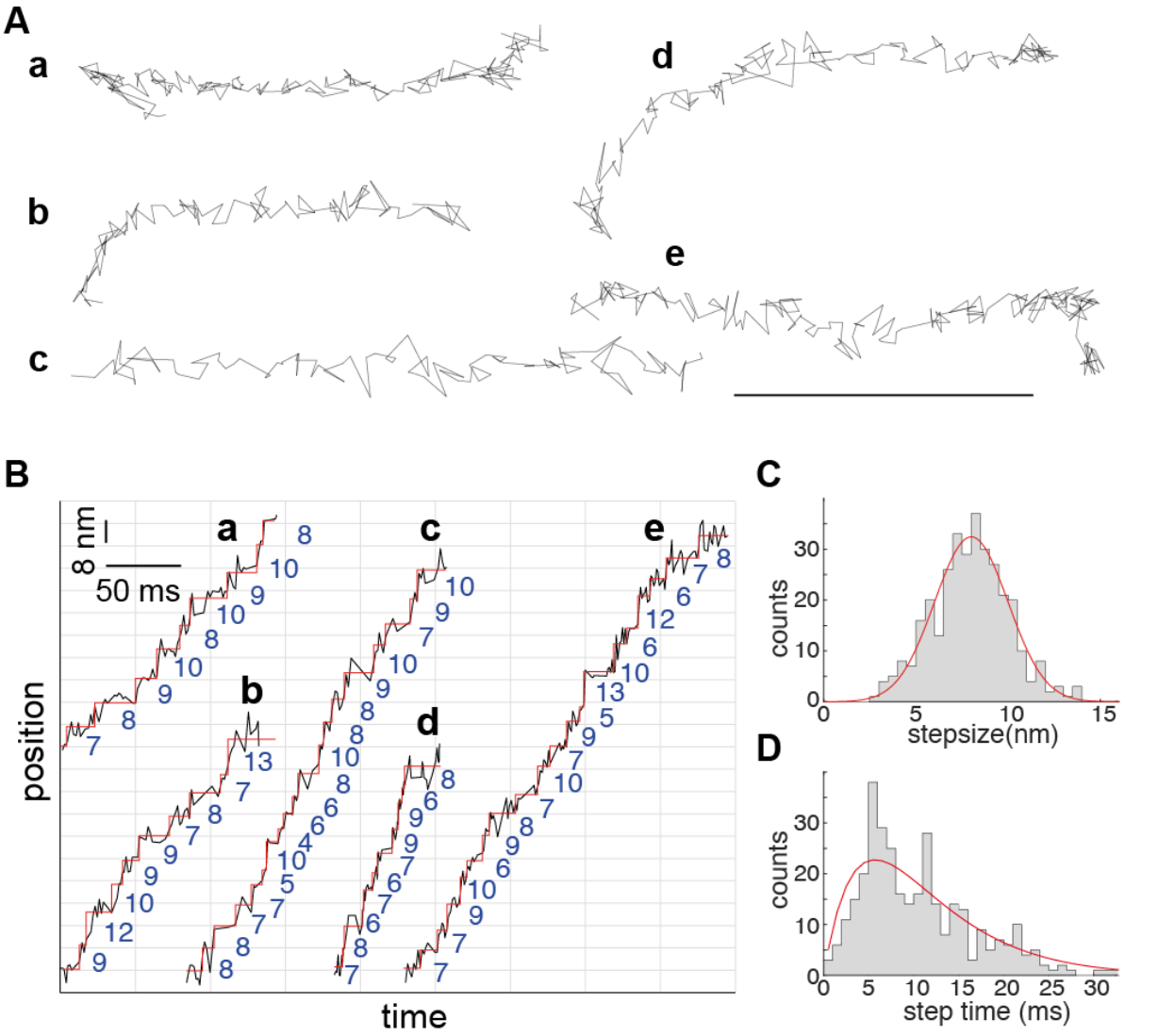

Fig. S7: Example trajectories of C-terminally labelled kinesin-1. (**A**) Representative tracks of K560-HaloTag labelled with JFX646-HaloTag ligand captured by 2D MINFLUX tracking in live U2OS cells are shown as line plots connecting each localization. (**B**) Corresponding time versus position plots, showing walking steps of around 8 nm. (**C**) Step size and (**D**) dwell time histograms with a fitted step size of 8.0 ± 2.8 SD ± 0.15 SEM nm and an average dwell time of 11.4 ms (data from 8 experiments, 21 tracks, and 334 steps). Scale bars: 100 nm.

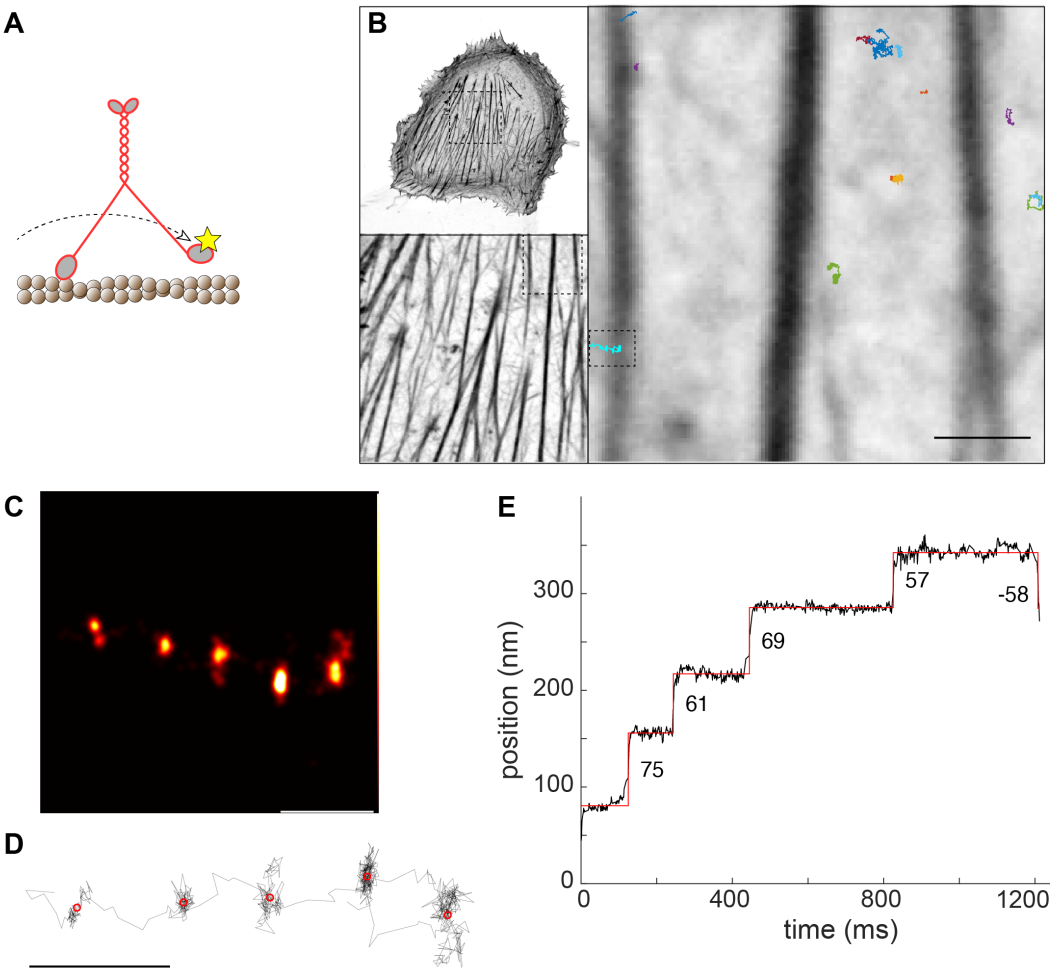

Fig. S8: Myosin-V tracking in live U2OS cells. (**A**) HaloTag-myosin-V[K1701A] walks in a hand-over-hand manner with a step size of 74 nm. (**B**) Confocal images of actin-GFP in a U2OS cell, and overlaid trajectories of human myosin-V (K1701A mutant), labeled with JF646 HaloTag ligand. (**C**) A myosin-V track where the localizations are rendered as a super-resolution image. (**D**) A line plot connecting each localization, with steps indicated in red. (**E**) Time versus position plot of the track, showing walking steps of around 74 nm. Scale bars: 100 nm (D), 1 µm (B).

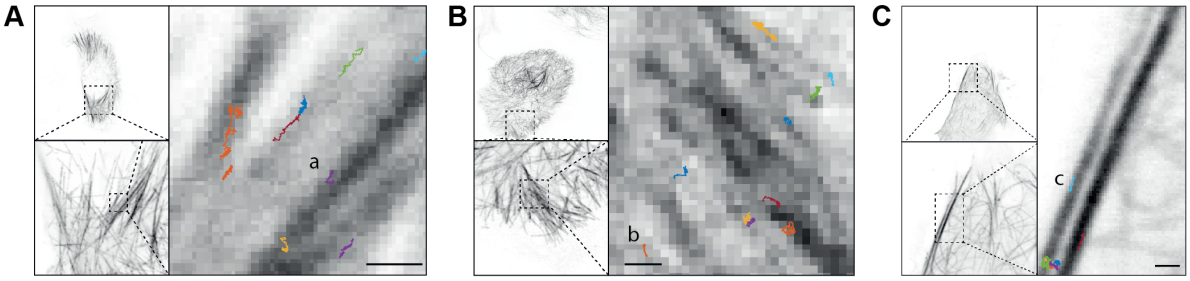

Fig. S9: Confocal images of live cells for 3D tracking. (**A, B, C**) Confocal images of GFP-α-tubulin in live cells where 3D tracks were captured. Tracks (a, b, c) correspond to tracks in Fig. 3 E, F. Scale bars: 500 nm (A, B, C).

	Localization precision (mean ± SD ± SEM) (nm)	Step size (mean ± SD[1] ± SEM[2]) (nm)	$1/k_1$ (ms)[3]	$1/k_2$ (ms)[3]	Dwell time from fit[4] (ms)
Motor-PAINT high ATP (Low laser, PFA fixed)	x: 2.2 ± 0.8 ± 0.01 y: 2.2 ± 0.9 ± 0.01	N.A.[5]	N.A.[5]	N.A.[5]	N.A.[5]
Motor-PAINT high ATP (High laser, PFA fixed)	x: 2.2 ± 0.5 ± 0.02 y: 2.3 ± 0.6 ± 0.02	7.8 ± 2.7 ± 0.09	22.6	8.3	30.8
Motor-PAINT low ATP (High laser, PFA fixed)	x: 2.2 ± 0.4 ± 0.02 y: 2.3 ± 0.3 ± 0.02	8.0 ± 2.2 ± 0.11	90.1	7.7	97.1
FL WT kinesin-1	x: 3.1 ± 1.4 ± 0.09 y: 2.7 ± 0.9 ± 0.06	15.7 ± 3.8 ± 0.25	23.1	0.6	46.8
K560 in U2OS cells (N-terminally tagged)	x: 3.2 ± 1.0 ± 0.02 y: 2.8 ± 0.9 ± 0.02	16.2 ± 3.8 ± 0.07	7.6	6.3	27.5
K560 in U2OS cells (C-terminally tagged)	x: 3.2 ± 1.4 ± 0.07 y: 3.1 ± 1.7 ± 0.09	8.0 ± 2.8 ± 0.15	5.7	5.7	11.4
K560 in neurons (N-terminally tagged)	x: 3.5 ± 1.4 ± 0.08 y: 3.0 ± 1.2 ± 0.06	15.7 ± 3.7 ± 0.21	7.4	7.3	29.2
K560 3D in U2OS cells (N-terminally tagged)	x: 4.3 ± 2.2 ± 0.14 y: 4.1 ± 1.9 ± 0.12 z: 3.4 ± 1.4 ± 0.09	16.4 ± 5.5 ± 0.37	12.9	0.4	26.3

Table S1: Experimentally obtained values of kinesin stepping dynamics. [1]Standard deviation (SD) from a Gaussian fit to the step size histogram. [2]Standard error of the mean (SEM). [3]Inverse of the rate constants from the fit. [4]An average dwell time calculated by fitting the histograms. See Materials and Methods for details. [5]Due to the low laser power and resulting low temporal resolution, kinesin steps were not resolved and thus these data are not available.

	Velocity[6] (mean ± SD) (nm/s)	Run length (mean ± SD) (nm)	Sample size[7]	Stalling[8]	Side-step[9] (%)
Motor-PAINT high ATP (Low laser, PFA fixed)	116 ± 75	555 ± 368	3 exp. 143 tracks N.A. steps[5]	81 % 3.6 /µm 2072 ms	19
Motor-PAINT high ATP (High laser, PFA fixed)	220 ± 68 (254)	252 ± 150	7 exp. 49 tracks 956 steps	61 % 4.2 /µm 965 ms	12
Motor-PAINT low ATP (High laser, PFA fixed)	81 ± 54 (82)	138 ± 85	5 exp. 45 tracks 393 steps	42 % 3.1 /µm 578 ms	2
FL WT kinesin-1	259 ± 177 (335)	239 ± 97	6 exp. 10 tracks 234 steps	Not observed	20
K560 in U2OS cells (N-terminally tagged)	523 ± 219 (591)	252 ± 163	161 exp. 330 tracks 2887 steps	2.4 % 0.1 /µm 591 ms	5.5
K560 in U2OS cells (C-terminally tagged)	706 ± 153 (704)	191 ± 80	8 exp. 21 tracks 334 steps	Not observed	4.8
K560 in neurons (N-terminally tagged)	437 ± 205 (538)	221 ± 102	10 exp. 23 tracks 303 steps	8.7 % 0.4 /µm 444 ms	4.3
K560 3D in U2OS cells (N-terminally tagged)	530 ± 254 (623)	365 ± 189	18 exp. 26 tracks 216 steps	Not observed	Not observed

Table S2: Experimentally obtained values of kinesin walking dynamics. [6]The number in the bracket is the velocity as calculated by multiplying the mean dwell time from the fit with the step size. [7]The sample size from each condition in the number of experiments, tracks, and steps. Each experiment is a biological replicate. [8]The number of tracks with stalling events longer than 300 ms were counted and presented relative to the total number of tracks. The number of occurrences per micrometer and the average stall time (in milliseconds) are also shown. [9]The number of tracks showing side-stepping (defined as a displacement of more than 6 nm in the direction perpendicular to the walking direction) as a percentage of the total number of tracks.

Movie S1.

Kinesin (*Dm*KHC (1-421)-SNAP-tag-6xHis) moving in a motor-PAINT U2OS cell at saturating ATP concentrations, acquired by a standard fluorescence microscope. Fig.1E-H. 250 frames with exposure time of 50 ms acquired every 100 ms.

Movie S2.

Kinesin (*Dm*KHC (1-421)-SNAP-tag-6xHis) track in a motor-PAINT glutaraldehyde-fixed U2OS cell showing side-stepping. Fig.1G3. Scale bar 10 nm.

Movie S3.

Kinesin (*Dm*KHC (1-421)-SNAP-tag-6xHis) track in a motor-PAINT U2OS cell at low ATP concentration. Fig.1L. Scale bar 10 nm.

Movie S4.

Kinesin (*Dm*KHC (1-421)-SNAP-tag-6xHis) track in a motor-PAINT U2OS cell at low ATP concentration showing zigzag motions. Fig.1M. Scale bar 10 nm.

Movie S5.

Full-length kinesin-1 (HaloTag-KIF5B) motilities in a live U2OS cell, acquired by a standard fluorescence microscope. Fig.2A-D. 900 frames with exposure time of 30 ms. Note that for MINFLUX tracking, we selected cells with lower kinesin expressions than in this video, exhibiting only several kinesins within the field of view.

Movie S6.

Full-length kinesin-1 (HaloTag-KIF5B) track in a live U2OS cell showing clear walking steps and side-stepping. Fig.2C. Scale bar 10 nm.

Movie S7.

Full-length kinesin-1 (HaloTag-KIF5B) track in a live U2OS cell showing back slipping and side-stepping. Fig.S4B3. Scale bar 10 nm.

Movie S8.

Kinesin (HaloTag-K560) motility in a live U2OS cell with Taxol treatment, acquired by a standard fluorescence microscope. Fig.2E-J. 1000 frames with exposure time of 30 ms. Note that for MINFLUX tracking, we selected cells with lower kinesin expressions than in this video, exhibiting only several kinesins within the field of view.

Movie S9.

Kinesin (HaloTag-K560) track in a live U2OS cell with Taxol treatment, showing clear walking steps. Fig.2Gd. Scale bar 10 nm.

Movie S10.

Kinesin (HaloTag-K560) 3D track in a live U2OS cell with Taxol treatment acquired by 3D MINFLUX tracking. Fig.3Ec.