Single particle trajectories reveal active endoplasmic reticulum luminal flow

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The endoplasmic reticulum (ER), a network of membranous sheets and pipes, supports functions encompassing biogenesis of secretory proteins and delivery of functional solutes throughout the cell. Molecular mobility through the ER network enables these functionalities, but diffusion alone is not sufficient to explain luminal transport across supramicrometre distances. Understanding the ER structure–function relationship is critical in light of mutations in ER morphology-regulating proteins that give rise to neurodegenerative disorders. Here, super-resolution microscopy and analysis of single particle trajectories of ER luminal proteins revealed that the topological organization of the ER correlates with distinct trafficking modes of its luminal content: with a dominant diffusive component in tubular junctions and a fast flow component in tubules. Particle trajectory orientations resolved over time revealed an alternating current of the ER contents, while fast ER super-resolution identified energy-dependent tubule contraction events at specific points as a plausible mechanism for generating active ER luminal flow. The discovery of active flow in the ER has implications for timely ER content distribution throughout the cell, particularly important for cells with extensive ER-containing projections such as neurons.

The endoplasmic reticulum (ER) is a contiguous network of membranous sheet-like reservoirs and tubes extending throughout the cell. Maintained by membrane-shaping proteins,2–4 this morphology supports the distribution of ER luminal content to distant sites. The ER-content distribution rate affects the efficiency of ER-mediated intracellular connectivity. Perturbation of this fundamental process may contribute to diseases caused by mutations in ER-shaping proteins.5,6 Measurements of ER-luminal protein mobility using fluorescence recovery after photobleaching (FRAP) have previously uncovered an energy dependence that is difficult to reconcile with passive diffusion.6 An increase in luminal crowding due to the incapacitation of ATP-dependent ER chaperones has been suggested as a plausible explanation for this effect (direct crowding measurements here do not detect such an effect). Addressing this paradox remained challenging since FRAP measurements report on bulk mobility, and do not inform as to the nature of forces driving mobility at a molecular level. A passive diffusion model for luminal transport is also challenged by the notion that traversal time of single-molecule displacement events. Using live-cell super-resolution microscopy, we visualized and analyzed single molecule trajectories traversing tubular ER, and the organelle’s real-time morphological dynamics.

First we examined, in our experimental system, the energy dependence of luminal protein mobility, previously demonstrated for green fluorescent protein (GFP) using FRAP. The escape rate of photoconvertible fluorescent protein (Dendra2-ER) from a region of activation was attenuated by ATP depletion (Fig. 1a). This may reflect either an increase in resistance to motion or a decrease in active transport of proteins on energy starvation. The former is contradicted by measurements of ER crowdedness, using a sensitive fluorescence resonance energy transfer (FRET)-based probe,7 that, over a broad range of expression levels, remained unaltered by ATP depletion (Fig. 1b–d and Supplementary Fig. 1a). Furthermore, displacement of Dendra2 proteins (initially localized as a ‘packet’ in a small volume of the tubular ER that had been subjected to a colour-switching laser pulse) occurred with variable speed and had a conspicuous unidirectional component (Fig. 1e and Supplementary Video 1). These features are inconsistent with Brownian motion and suggest, instead, active transport.

To characterize motion in the ER lumen, we performed real-time SPT in live cells, acquiring trajectories at optical super-resolution. Imaging at 561 Hz, we recorded single molecule fluorescent signals from functional (Halo-tagged ER luminal chaperone, calreticulin, Crt) and inert (ER-targeted HaloTag) proteins, both sparsely labelled with a fluorescent ligand (chloroalkane–tetramethylrhodamine, TMR). The brightness and photostability of the TMR ligand enabled the tracking of single particles over longer trajectories than previously available in bulk methods such as FRAP. By implementing a single molecule localization algorithm, images reconstructed from the SPT series revealed a pattern typical of the ER network, confirming ER-localization of the HaloTag–Crt and washout of the unbound dye (Fig. 2a,b and Supplementary Videos 2–4).

Trajectories were generated from single-molecule time series by sequentially implementing spot detection and tracking algorithms.8 ER geometry constrains particle movement to a narrow
tubular network, which limits overlapping trajectories, contributing to the tracking algorithm’s ability to faithfully trace many molecules simultaneously. Resulting trajectories reconstruct a map recognizable as a pattern of ER tubes and their connecting reservoirs (Fig. 2c), reflecting tracking fidelity. Notably, spatio-temporal particle distributions were non-uniform, with higher time-integrated abundance in the tube-connecting reservoirs (Fig. 2d). This heterogeneity correlated with spatial distribution of instantaneous velocities, revealing distinct subgroups: relatively slow-moving particles predominantly detectable in segments of trajectories mapped to the nodes; and particles with relatively high and variable velocities mapped to node-connecting tubes (Fig. 2c). These characteristics could be observed for two different markers (Crt and HaloTagER) and three different cell types (HEK-293T, COS-7 and SH-SY5Y, Supplementary Table 1). The long, single-tailed velocity distribution observed (histogram, Fig. 2c) is incompatible with Brownian...
**Fig. 2 | Characteristics of single particle displacement tracked in the tubular ER lumen.** **a,** An image reconstructed from single molecule localizations of TMR-labelled Halo-tagged Crt, in HEK-293 cells, rendered with a molecular density colour code. **b,** A skeletonization view of the image in **a.** Shown are representatives of n = 3 independent experiments. **c,** Single molecule trajectories generated using a particle-tracking algorithm from the time series of image **a,** colour-coded according to the instantaneous velocity distribution shown in the histogram. Overlaid traces: the velocity distribution simulated assuming exclusively diffusion-driven motion (solid line, using apparent D from **f**), or a combination of diffusion and flow (using D and flow rate from **f** and **g**). Inset: cumulative distribution, Kolmogorov–Smirnov test of observed versus expected distributions. **d,** A density map computed for a grid of square bins (sides of 0.2 μm) imposed on the particle displacement map. The ellipses mark boundaries of higher-density regions (correspond to tube-connecting reservoirs/junctions). **e,** Histograms of instantaneous velocity frequency distributions of SPT from a cell before/after ATP depletion (as in Fig. 1a–c). Inset: a violin plot presenting the medians (red bars) and density (grey) of the distributions. A two-sided Mann–Whitney U-test was used to compare the median of each pair of distributions (*****P < 1 × 10^{-3} ), P_{(0–20 min)} = 1 × 10^{-89}, P_{(20–40 min)} = 9.889 × 10^{-141}, P_{(0–40 min)} = 1 × 10^{-92}; n = 20,526, n = 14,591 and n = 10,108 trajectory displacements respectively. **f,** A diffusion map extracted from the empirical estimator of the Langevin equation (equation (1), see computation details in Supplementary Note 1) and computed from a square grid as in **d.** Inset: distributions of the diffusion coefficients inside nodes (avg ± s.d. = 0.19 ± 0.13, n = 226 nodes). **g,** Flow map computed by averaging non-Brownian velocity jumps of particles moving between pairs of neighbouring nodes identified in **d** and colour-coded according to the inset histogram. Inset: the distribution of the average instantaneous velocity between pairs of neighbouring nodes (n = 705 node-pairs; avg ± s.d. = 22.90 ± 6.92). Raw source single molecule time series and image reconstruction are shown in Supplementary Video 2.
motion (Fig. 2c, modelled by a solid line). Furthermore, ATP depletion led to selective loss of the fast-moving population (Fig. 2c). These observations suggest that diffusive motion is manifested by the slow-moving particles in the tube-connecting reservoirs (nodes), while the rapidly moving particles in the tubes are subjected to an ATP-dependent propulsive force, resulting in an ER luminal flow. The displacement profile observed fits well a bi-modal distribution of instantaneous velocities (Fig. 2c, modelled by a dashed line).

Next we quantified temporal coordinate changes of HaloTag–Crt by analysing SPT data using the overdamped limit of the Langevin model (where velocity is described as the sum of diffusional and drift forces, and motion parameters are estimated from local statistics of the displacement, see Methods)\(^\text{10-13}\). Motion is described by the stochastic model (a sum of directed and diffusional motion terms):

\[
\dot{X} = b(X) + \sqrt{2D(X)} \Psi
\]

where \(\Psi\) is Brownian motion. The source of the noise \(\Psi\) is the ambient thermal agitation, while the drift term \(b\) represents transport in tubules and \(D\) is the effective local diffusion. This analysis (see Supplementary Note 1) allows the estimation of \(D\) and \(b\) from large numbers (~10\(^4\) per cell acquisition) of single particle trajectories.

The global nature of this statistical approach considers not only particle displacement speed but also direction patterns, extracted from a large number of trajectories repeatedly traversing the same regions, thereby unmixing the contribution of Brownian and deterministic forces. This computation identifies a slow diffusional \((D = 0.19 \pm 0.13 \mu m^2 s^{-1})\) component that maps predominantly onto the nodes (Fig. 2f). The relatively fast movement of internode particles required an additional component to account for their directionality and persistence, consistent with a propulsive force with normal distribution of velocity \((22.9 \pm 6.92 \mu m s^{-1})\, \text{Fig. 2g})\).

The super-diffusive nature of particle motion in ER tubules was further confirmed by analysis of time-averaged mean squared displacement (MSD) of SPT. The time-averaged MSD can be described by \(\sim r^\alpha\), where the anomalous exponent \(\alpha\) defines motion as sub-diffusive if \(\alpha < 1\), Brownian/diffusive if \(\alpha = 1\) and super-diffusive if \(\alpha > 1\). Conducted on the entire ensemble of trajectories, the MSD analysis revealed a broad range of particle behaviours \((0 < \alpha < 1.5, \text{Fig. 2b})\), whereas the same analysis restricted to trajectory fragments located in nodes revealed clearly confined diffusion dynamics \((\alpha < 0.8, \text{Supplementary Fig. 2c})\). Trajectories of particles moving outside the nodes exhibited super-diffusive dynamics \((\alpha > 1, \text{Supplementary Fig. 2d and Supplementary Video 3})\). These
results are consistent with the active motion mode identified in the analysis of Fig. 2.

Similar observations were made in green monkey kidney (COS-7) and human neuroblastoma (SH-SY5Y) cell lines, attesting to their broad validity in describing ER flow dynamics and its spatial organization (Supplementary Fig. 3, Supplementary Table 1 and Supplementary Video 2). Measurements of the motion parameters of a lower-mass ER-localized protein, HaloTag–KDEL, showed similar values to those observed with the tagged Crt (Supplementary Fig. 3 and Supplementary Table 1). Flow velocity was slightly higher in COS cells than in HEK-293T and SH-SY5Y cells. The behaviour of the luminal ER markers, HaloTag–KDEL and Crt, contrasted with that of a membrane-associated analogue of the latter, mEOS2–calnexin: its velocities were distributed relatively homogeneously through the ER network (Fig. 3a,b), lacked the thick tail of high values in distribution of instantaneous velocities and fitted well to a purely diffusional model (Fig. 3a–d).

To establish whether recently reported ER macrostructure motion characteristics14 are reflected in the SPT analysis, we focused on their numerical parameters. Motion of ER tubules characterized by their relatively slow transverse oscillation (4 Hz, with an amplitude <50 nm, which translates to velocity <0.2 μm s⁻¹; ref. 14), does not significantly contribute to the relatively fast velocities of flow-assisted marker particles moving along the tubules (27–42 μm s⁻¹, Supplementary Table 1). Junction fluctuations contribute a similarly insignificant component to the diffusional motion inside the junction since the diffusion coefficient calculated from tracking of whole junctions was 69 times slower than the mean diffusion coefficient computed for single molecules14 (Supplementary Fig. 4 and Supplementary Video 5). Furthermore, contribution of tubule growth to single particle trajectories was found to be negligible,
**Fig. 5 | Dynamics of ER luminal flow correlated with tubule contractions.** a. Analysis of particle trajectories’ directionality. Tubular junctions/nodes are denoted by orange ellipses; grey lines denote all particle trajectories. Trajectories connecting two nodes indicated as A and B are colour-coded according to their direction either in red (B to A), or blue (A to B). The lower graph represents the temporal pattern of traversing-directionality. The results shown are representative of n = 108 node-pairs. b. Distribution of time periods of unidirectional inter-node displacement. c. Plots of instantaneous particle velocities fluctuations. Velocities of particles following departure from nodes and travelling along tubules (between nodes, red), and those of particles residing within nodes (black). The solid lines represent mean values for all trajectories, and shaded regions represent s.d. of total sample size: n = 111 internode and n = 140 intranode trajectory displacements. d. Analysis of time duration $T_D$ of high-velocity ($v > 20 \mu m s^{-1}$) peaks (left) and time interval $T_I$ (right) between high-velocity peaks. Insets denote $T_D$ and $T_I$ of a trajectory. The red line represents an exponential fit ($R^2 = 0.998$). e. High-speed SIM super-resolved images of the tubular ER in live COS7 cells stained with an ER membrane dye (ER tracker green). Images were acquired in 54 ms intervals and processed as described in Methods. The resulting SIM reconstructions were colour-coded according to intensity. The magnified area shows the contours of ER tubules at higher magnification. The arrows denote positions where transient contraction events occur repeatedly. Shown are frames from a time series measurement presented in full in Supplementary Video 8. Tubule contractions are better visualized in COS7 cells, but detectable in HEK-293 cells too (Supplementary Fig. 5). The results shown are representative of n = 5 independent experiments. f. Box plot of tubule contraction frequencies extracted from high-speed SIM time series as shown in e before and after ATP depletion. Red line, median; boxes’ bottom/top edges, the 25th and 75th percentiles, respectively; whiskers, extreme data points. Two-sided Mann–Whitney U-test $P = 1.7019 \times 10^{-7}$, n = 20 ER tubules. g. Distributions of contraction duration, intervals and lengths from SIM videos as in e and Supplementary Fig. 5. Red curves: exponential (left and middle) and Gaussian (right) fits ($R^2 = 0.988$, $R^2 = 0.969$, $R^2 = 0.937$, respectively). h. Schematic representation of the model for estimating tubule contraction-induced particle velocity. All values are given as avg ± s.d. for the noted n.
with a mean percentage of tubes growing at any given time of 0.14 ± 0.04% (Supplementary Videos 5–7).

Consistently, considering trajectory motion as a purely diffusive process yielded an apparent diffusion coefficient of 1.13 μm²·s⁻¹ (Supplementary Fig. 2a) similar to that previously estimated by FRAP⁴,⁵,⁶. Although most individual trajectories visited only a limited number of nodes (Fig. 4a), an oriented network graph analysis, which identifies directly or proxy interconnected junctions through trajectory directions⁷, revealed that, regardless of their starting point, particles have the potential to visit almost the entire ER network (Fig. 4b; the disconnected periphery is probably contributed by signals from neighbouring cell(s)). This analysis is consistent with the notion that the ER network maintains a luminal continuum. The ER appeared to be in a state of equilibrium, with nodes, on average, connected by an equal number of inward and outward trajectories (Fig. 4c,d). These findings are consistent with an interconnected system of flows that preserves the content across the ER.

Closer scrutiny of the directionality of individual tube-traversing particles suggested a pattern whereby the direction of visible flow alternates with variable frequency (switching on average every 4 s, maintained for up to 14 s, Fig. 5a,b); and particles accelerated periodically following their exit from a node, reaching brief velocity peaks that lasted up to 120 ms (Fig. 5c,d). Intervals between velocity peaks and flow-directionality alternations were distributed stochastically (Fig. 5b,d), suggesting that flow-inducing events (for example, transient tubule contractions, discussed below) are not produced by synchronized oscillators and are therefore not centrally coordinated. However, we cannot exclude the contribution of synchronization processes whose phase is lost, as trajectories are recorded asynchronously. Note that temporal profiles of directionality are not available using low spatio-temporal resolution approaches (for example, FRAP or photoconversion pulse-chase, Fig. 1e).

The oscillatory luminal motion suggested the possibility of nanoperistalsis-like propulsion, attainable by tubule contractions. To test this, we obtained high-resolution time-series images of the ER tube structure of live cells by fast structured illumination microscopy (SIM)⁸,⁹. These revealed transient, asynchronous constriction of the tubes at specific locations (Fig. 5e, Supplementary Fig. 5a,b and Supplementary Videos 8 and 9), consistent with a role for tube constriction in generating flow. Constriction-driven propulsion is also consistent with the observed velocity values and variation of packets of photoconverted Dendra2 during their deterministic traversal of the tubular ER (Vmean = 19.9 μm·s⁻¹, Fig. 1e). Furthermore, the frequency of contraction events decreased four-fold on ATP depletion (Fig. 5f and Supplementary Video 10). It is expected that following an individual contraction event (with a frequency of hundreds of milliseconds), both deterministic and acceleratory displacement of multiple particles would be detected, as PET acquisition operates at approximately ten times the contraction frequency. Assuming uncoordinated contractions throughout the tubular network, consecutive contractions have the same probability to preserve or invert the direction of the next set of detectable SPT events (consistent with observed distribution of directionality preservation time, Fig. 5b).

The notion that tubule contractions generate high-velocity peaks in luminal particles is supported by the fact that their temporal distributions are both Poissonian (Fig. 5d,g), indicating compatible physical processes. The larger time constant of contractions (~900 ms) compared to that of high-velocity peaks (~80 ms) is expected since several contraction points may contribute to the particles’ acceleration incidence.

Furthermore, a physical model simulating forces resulting from tubule contraction, and based on their empirical characteristics (Fig. 5b and Supplementary Note 1), predicts flow velocities of 10–40 μm·s⁻¹, in agreement with the high velocities observed in SPT (Fig. 5c). Notably, the contraction frequency is low enough to avoid coinciding proximal contractions that may cancel the local flows (probability of simultaneous contractions of two points = 0.022, calculations in Supplementary Note 1). While the existence of a mechanism for spatio-temporal coordination of the contraction events cannot be ruled out, our findings indicate that an uncoordinated system, inducing fast local currents with alternating directionality inside the tubular network, is sufficient to ensure a rapid luminal content homogenization/distribution and thereby overcome a critical kinetic limitation of passive diffusion as a mechanism for ER content mixing in large cells.

Localized contraction of ER tubules, leading to ER deformation, was observed during calcium manipulation (Supplementary Fig. 5c), or phototoxicity (Supplementary Fig. 5e and Supplementary Video 11), both reversible processes affecting ER morphology (Supplementary Fig. 5e). These super-resolved images of the ER’s structural dynamics under severe experimental perturbation highlight a potential for ER tubes to contract, revealing that fragments of the perturbed ER that had lost their tubular structure displayed characteristic slow-velocity diffusional motion (Supplementary Fig. 5d). Other physiological membrane dynamic processes involving molecular motors, vesicular fusion and budding, network oscillations and even tube elongation/withdrawal may also contribute to flow and warrant further investigation.

Regardless of their origin(s), the alternating luminal currents described here are well suited to serve as a mixing device, enhancing distribution of ER content throughout the cell. Given that diffusion-driven connectivity (matter exchange rate) decreases exponentially with distance, it is expected that the active process described here would be especially important in cells with extensive ER projections, such as motor neurons. It is therefore tempting to speculate that perturbed luminal flow might contribute to diseases such as hereditary spastic paraplegia, associated with defective ER membrane-shaping proteins.

Online content
Any methods, additional references, Nature Research reporting summaries, source data, statements of data availability and associated accession codes are available at https://doi.org/10.1038/s41556-018-0192-2.

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Author contributions

D.H. and P.P. designed, executed and interpreted the mathematic modelling and analysis of S.P.T. P.P. designed and created the custom code for the data analysis. J.E.C. executed and analysed the FLIM experiments. S.J.M. contributed to the FLIM design and analysis. M.F. and L.J.Y. developed the fast SIM system, acquired SIM images and developed custom code for image processing and analysis. C.F.K. contributed to FLIM and SIM design, analysis and interpretation. D.R. oversaw the project, contributed to the design and interpretation of the experiments, and edited the manuscript. E.A. conceived and led the project, executed analysed and interpreted cell structured illumination and single particle imaging, and contributed to the image data processing. E.A. and D.H. designed the project and wrote the manuscript. All authors read and approved the final manuscript.

Competing interests

The authors declare no competing interests.

Additional information

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Methods

Intra-vital and fixation cell microscopy. Cell culture, transfections and expression constructs. COS7 (RRID:CVCL_0224) and HEK-293 cells were cultured in Dulbecco’s modified Eagle’s medium (DMEM) supplemented with 10% fetal calf serum and 1× non-essential amino acids (M7145, Sigma). SH-SY5Y cells were cultured in MEM/F12 medium supplemented with 15% fetal calf serum. Transfections were performed using the Neon Transfection System (Invitrogen) applying 1.5 μg of ATeam or crowding probe DNA to 3×10^6 cells. An expression vector encoding a cytosol-localized crowding probe was modified to encode an ER-localized probe by amino-terminal addition of mouse preprotrypsin signal sequence and carboxy-terminal addition of a KDEL motif by Gibson assembly. Descriptions of the plasmids used in this study are presented in Supplementary Table 2.

Photoconversion microscopy. Photoconversion pulse-chase experiments were performed using a Leica SP8 confocal microscope. Images were acquired using a frame size of 256×256 pixels to allow imaging at 9 frames s\(^{-1}\) rate, in the green (488 nm excitation, 510–530 nm emission) and red (561 nm excitation, 590–620 nm emission) channels. Photoconversion illumination (405 nm) was introduced at frame 20 in a region of interest for a duration of 20 frames, using On The Fly FRAP acquisition mode (enabling image recording during the photocovertion illumination). Normalized intensity of the red channel at the region of photoconversion was plotted as a function of time post-photoconversion, and fitted to a mono-exponential decay function, to extract the decay \(t_{\text{dp}}\), which was used as an estimator of Dendra2-ER mobility.

Cell manipulations and fluorescence lifetime imaging microscopy. Fluorescence lifetime imaging microscopy was carried out as previously described\(^{24,27}\), using a pulsed (sub-10 ps, 20–40 MHz) supercontinuum (430–2000 nm) light source (SC 450, Fianium Ltd). An acousto-optic tunable filter (AA Optoelctronic AOTFnc-Vis) was used to define the 438 nm excitation wavelength for both the ER-crowding probe and the ATeam ATC sensor. Emitted light was collected using a 470/20 nm emission filter and detected by a fast photomultiplier tube (PMF-100, Becker & Hickl GmbH). Data were processed using SPImage (Becker & Hickl GmbH), fitting a monoexponential decay function. Osmosis-driven changes in cell volume were induced by addition of 0 mM NaCl (hypo-osmotic) or 500 mM NaCl (hyperosmotic) supplemented Hanks balanced salt solution (2.5 mM KCl, 1.2 mM CaCl\(_2\), 0.5 mM MgCl\(_2\), 5 mM glucose, 10 mM HEPES pH 7.5) to a final ratio of 1:1 with DMEM (final osmolalities of 0.152 osmol kg\(^{-1}\) and 0.609 osmol kg\(^{-1}\) respectively). ATP depletion was achieved by incubating the cells in the presence of NaN\(_3\) (0.05% w/v) and 2-deoxy-glucose (20 mM) for 2 h before imaging; cells preserving the tubular ER pattern were selected following treatments.

Single particle localisation and tracking. Cells were transiently transfected with vectors encoding HaloTag (Promega) targeted to the ER by an N-terminal preprotrypsin signal sequence and a C-terminal retrieval signal (SS–HaloTag–KDEL); or HaloTag–Crt. At 24 h after transfection, cells were labelled with 0.5 mM cell-permeant HaloTag–TMR ligand (Promega GR251) for 10 min, followed by 3 washes with label-free medium; and imaged with 18 ms exposure on an Elyra Super Resolution microscope (Zeiss) using an α-Plan Apochromat x100 oil, 1.46 NA objective at 561/570–620 nm excitation/emission, in HiLo (pseudo-TIRF) mode using an EMCCD iXON DU897 camera (Andor). The obtained single particle image series (at least 2,000 frames) were processed using the PALM image reconstruction module or particle-tracking module of the instrument’s software (Elyra Zen edition, Zeiss). Trajectory generation fidelity was verified using ICY software\(^{26}\) version 1.9.5 and Iinaris software version 8.4.1 (Bitplane).

The tracking algorithm was set to identify diffraction-limited spots as particles if their signal-to-noise ratio was >4 and the spot did not exceed 9 pixels in diameter; then to identify the centres of spots to refine the positioning of the particle beyond the diffraction limit. The algorithm terminated trajectories if the signal disappeared for 1 frame (linking is not permitted if particles leave the focal plane, blank or so on).

The tracking stage produces \(N_t\) two-dimensional trajectories \(X_{t_{1}} \ldots X_{t_{M}}\) each possessing \(M\) points: \(X_{t_{i}} = (x_{t_{i}}, y_{t_{i}})\) (1 ≤ \(i\) ≤ \(N\), 0 ≤ \(t_{i}\) ≤ \(T\)). Trajectories containing fewer than three points were discarded from the analysis. For each pair of successive points \((t_{i}, t_{i+1})\) of a trajectory \(i\), we defined the displacement as: \(\Delta X_{t_{i}} = X_{t_{i+1}} - X_{t_{i}}\). See Supplementary Note 1 for further details of SPT mathematical analysis and modelling.

High-speed structured illumination microscopy. Live two-dimensional SIM (light modulation/grazing incidence illumination microscopy, GI-SIM) images of cells stained for 20 min with ER tracker green (ThermoFisher Scientific, E34251) were acquired with a custom-built high-speed SIM microscope\(^{25}\), using a spatial light modulator. ER tracker green was imaged using a 100×/1.49 NA TIRF oil-immersion objective (Olympus) with a 488 nm diode laser (Topica) at an irradiance of 50 W cm\(^{-2}\), with emission imaged via a notch filter (FF01-525/30, Semrock) onto an sCMOS camera (ORCA Flash 4.0, Hamamatsu). Two-dimensional SIM gratings displayed on the spatial light modulator resulted in a linear spacing of 228 nm at the sample, corresponding to an angle of incidence of 44.6°. Each super-resolved frame was obtained from the reconstruction\(^{26,27}\) of 9 raw frames acquired at 6 ms per exposure (54 ms per SIM frame). 3D-SIM (23 slices, 2.4 μm) was performed using an Elyra microscope (Zeiss), with a Plan Apochromat x63/1.4 NA objective and an sCMOS PCO Edge camera (Andor) on paraformaldehyde 2%, gluteraldehyde 2%, 100 mM sodium cacodylate, 2 mM CaCl\(_2\), pH 7.4 for 1 h at room temperature. See Supplementary Note 1 for further details of SIM image analysis.

Mathematical modelling and analysis. Details of the computational analyses of SPT and SIM, and their mathematical modelling are described in Supplementary Note 1.

Statistics and reproducibility. Statistical analyses and visualization were performed using Matlab 9. Error bars, P values and statistical tests and sample sizes are reported in the figure legends. Statistical differences between probability distribution were assessed using two-way Kolmogorov-Smirnov tests and statistical differences between distribution medians were assessed using two-sided Mann–Whitney U-tests. All experiments were performed independently at least three times.

Reporting Summary. Further information on research design is available in the Nature Research Reporting Summary linked to this article.

Code availability. Custom code generated for SPT analysis and visualization as well as for SIM ER network analysis of SIM images can be obtained from the Zenodo database along with experimental raw data examples, https://doi.org/10.5281/zenodo.1317630 and https://doi.org/10.5281/zenodo.1318129, respectively.

Data availability

Source image-series data for Fig. 1e and Supplementary Figs. 2 and 4ef have been provided as Supplementary Videos 1, 2 and 8–10 respectively; and statistical information for Fig. 1e and Supplementary Fig. 2 have been provided in Supplementary Table 1. Custom code has been deposited in the Zenodo database (https://doi.org/10.5281/zenodo.1317630) with experimental raw data (https://doi.org/10.5281/zenodo.1318129), respectively.

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Reporting Summary

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Statistical parameters

When statistical analyses are reported, confirm that the following items are present in the relevant location (e.g. figure legend, table legend, main text, or Methods section).

| n/a | Confirmed |
|-----|-----------|
| ☑   | The exact sample size \((n)\) for each experimental group/condition, given as a discrete number and unit of measurement |
| ☑   | An indication of whether measurements were taken from distinct samples or whether the same sample was measured repeatedly |
| ☑   | The statistical test(s) used AND whether they are one- or two-sided |
| ☑   | Only common tests should be described solely by name; describe more complex techniques in the Methods section. |
| ☑   | A description of all covariates tested |
| ☑   | A description of any assumptions or corrections, such as tests of normality and adjustment for multiple comparisons |
| ☑   | A full description of the statistics including central tendency (e.g. means) or other basic estimates (e.g. regression coefficient) AND variation (e.g. standard deviation) or associated estimates of uncertainty (e.g. confidence intervals) |
| ☑   | For null hypothesis testing, the test statistic (e.g. \(F\), \(t\), \(r\)) with confidence intervals, effect sizes, degrees of freedom and \(P\) value noted |
| ☑   | Give \(P\) values as exact values whenever suitable. |
| ☑   | For Bayesian analysis, information on the choice of priors and Markov chain Monte Carlo settings |
| ☑   | For hierarchical and complex designs, identification of the appropriate level for tests and full reporting of outcomes |
| ☑   | Estimates of effect sizes (e.g. Cohen's \(d\), Pearson's \(r\)), indicating how they were calculated |
| ☑   | Clearly defined error bars |
| ☑   | State explicitly what error bars represent (e.g. SD, SE, CI) |

Our web collection on statistics for biologists may be useful.

Software and code

Policy information about availability of computer code

Data collection: Super-resolution image series were acquired using the Elyra Zen edition (Zeiss).

Data analysis: Single particle image series were processed using the PALM image reconstruction module of Elyra Zen edition (Zeiss). Trajectory generation fidelity was verified using ICY 1.9.5 and Imaris 8.4.1 (Bitplane) software. ImageJ 1.0 was used to render structured illumination image series. Matlab 9 (Mathworks) was used for trajectories analysis, as described in methods. Graph reconstruction from SPTs uses the DBSCAN algorithm implemented in scikit-learn obtained through the Anaconda distribution (Anaconda Inc.) v.4.3.8. Junction positions were determined and extracted from fSIM images using the AnalyzeSkeleton plugin from ImageJ 1.51 and junction trajectories were reconstructed with ICY 1.9.5. FLIM images were analysed using Backer and Hickl SPCI 7. Custom code generated for single particle tracking analysis and visualisation, as well as for ER network analysis of SIM images, can be obtained from Zenodo database along with experimental raw data examples, DOI: 10.5281/zenodo.1317630 and DOI: 10.5281/zenodo.1318129, respectively.

For manuscripts utilizing custom algorithms or software that are central to the research but not yet described in published literature, software must be made available to editors/reviewers upon request. We strongly encourage code deposition in a community repository (e.g. GitHub). See the Nature Research guidelines for submitting code & software for further information.
Data

Policy information about availability of data

All manuscripts must include a data availability statement. This statement should provide the following information, where applicable:
- Accession codes, unique identifiers, or web links for publicly available datasets
- A list of figures that have associated raw data
- A description of any restrictions on data availability

Source image-series data for Fig. 1e, Supplementary Fig. 2 and Fig 4e, f have been provided as supplementary video1, 2 and 8 - 10 respectively; and statistical information for Fig. 2 and supplementary Fig. 2 in supplementary table 1. All other data supporting the findings of this study are available from the corresponding authors on reasonable request. Custom code generated for single particle tracking analysis and visualisation as well as for SIM-ER network analysis of SIM images can be obtained from Zenodo database along with experimental raw data examples, DOI: 10.5281/zenodo.1317630 and DOI: 10.5281/zenodo.1318129 respectively.

Field-specific reporting

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Life sciences

For a reference copy of the document with all sections, see nature.com/authors/policies/ReportingSummary-flat.pdf

Life sciences study design

All studies must disclose on these points even when the disclosure is negative.

| Sample size | No statistical method was used to determine sample size. The sample size was sufficient to yield clear statistical significance. Maximal number of trajectories (tens of thousands) that can be handled using the available computational tool was sampled from the live cell time series. |
| Data exclusions | Cells with clear morphological appearance of necrosis/apoptosis in eye examination using light microscopy were excluded. For quality control purposes trajectories with less than three detection points were excluded from analysis, as described in methods. |
| Replication | All attempts at replication noted in figures were successful. All the reagents used are indicated, and the methods, including original techniques developed for the purpose of the current study, are described in detail. |
| Randomization | Cell for microscopy measurements were randomly selected from sample cultures in independent repeats. |
| Blinding | Mathematical analysis of most of the single particle trajectories data was performed by an investigator who was blind to the type of cell and organelle marker used in the analysed cells. |

Reporting for specific materials, systems and methods

Materials & experimental systems

| n/a | Involved in the study |
| ☒ | Unique biological materials |
| ☒ | Antibodies |
| ☒ | Eukaryotic cell lines |
| ☒ | Palaeontology |
| ☒ | Animals and other organisms |
| ☒ | Human research participants |

Methods

| n/a | Involved in the study |
| ☒ | ChIP-seq |
| ☒ | Flow cytometry |
| ☒ | MRI-based neuroimaging |
## Eukaryotic cell lines

Policy information about cell lines

| Cell line source(s) | HEK 293, COS-7 and SH-SY5Y cells used in the present study were from ATCC. |
|---------------------|------------------------------------------------------------------------------|
| Authentication      | Cell authentication was based on morphological criteria, in case of SH-SY5Y cells their ability to undergo differentiation into neurons was observed. |
| Mycoplasma contamination | Cells were periodically tested for mycoplasma contamination and found negative. |
| Commonly misidentified lines (See ICLAC register) | No commonly misidentified cell lines were used. |