Homogeneous multifocal excitation for high-throughput super-resolution imaging

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Super-resolution microscopies have become an established tool in biological research. However, imaging throughput remains a main bottleneck in acquiring large datasets required for quantitative biology. Here we describe multifocal flat illumination for field-independent imaging (mfFIFI). By integrating mfFIFI into an instant structured illumination microscope (iSIM), we extend the field of view (FOV) to >100 × 100 μm² while maintaining high-speed, multicolor, volumetric imaging at double the diffraction-limited resolution. We further extend the effective FOV by stitching adjacent images for fast live-cell super-resolution imaging of dozens of FOVs. Finally, we combine our flat-fielded iSIM with ultrastructure expansion microscopy to collect three-dimensional (3D) images of hundreds of centrioles in human cells, or thousands of purified Chlamydomonas reinhardtii centrioles, per hour at an effective resolution of ~35 nm. Classification and particle averaging of these large datasets enables 3D mapping of posttranslational modifications of centriolar microtubules, revealing differences in their coverage and positioning.
Results
Design and implementation of homogeneous multifocal excitation.
Generating a uniform irradiance over a large FOV while retaining the highest achievable resolution requires the following properties of the excitation focal spots in a multifocal microscope: (1) homogeneous intensity, (2) constant pitch and (3) diffraction-limited size across the FOV. Existing methods for generating uniform excitation are often limited to producing a low number of excitation spots, with residual heterogeneity and low transmission efficiency. Thus, we set out to extend the Köhler integrator\(^2\)\(^\text{2}\)\(^\text{2}\)\(^\text{2}\)\(^\text{2}\), which averages over the spatial and angular distributions of the light source, for use with multifocal excitation. The Köhler integrator is primarily composed of a rotating diffuser sampling different interference patterns, a pair of microlens arrays (MLAs) forming parallelized imaging channels and a Fourier lens averaging those channels into a flat-top (Supplementary Information and Supplementary Video 1)\(^2\)\(^\text{2}\)\(^\text{2}\)\(^\text{2}\)\(^\text{2}\).

Previously, multifocal excitation in iSIM was generated by illuminating a MLA with a collimated Gaussian beam\(^2\)\(^\text{2}\)\(^\text{2}\)\(^\text{2}\)\(^\text{2}\). As expected, the nonhomogeneous beam profile results in excitation points whose intensity depends on their location, as recapitulated in a wave optics simulation (Fig. 1a and Supplementary Information). Alternatively, illuminating the excitation MLA with a flat-top beam—such as that produced by a Köhler integrator—results in an array of multifocal excitation spots of homogeneous intensity (Fig. 1b). This is achieved by placing the excitation MLA at the front focal plane of the Fourier lens of the Köhler integrator, where the flat-top is focused (Extended Data Fig. 1). However, since the incident wavefront is not flat, the illumination produced by this configuration is nontelecentric and alters the pitch of the excitation spots in the focal plane of the excitation MLA (Fig. 1c). This periodicity—usually determined by the pitch of the excitation MLA—must match the periodicity of additional components that are typically placed in conjugate image planes of confocal microscopes, such as a pinhole array. In case of mismatch, the light will be gradually occluded and decrease in intensity away from the center of the optical path.

To solve this problem, we place the Fourier lens of the Köhler integrator one focal length away from the flat-fielding MLA\(^3\)\(^\text{3}\)\(^\text{3}\)\(^\text{3}\)\(^\text{3}\), resulting in a flat wavefront and thereby ensuring that the pitch of the excitation is conserved (Fig. 1d and Extended Data Fig. 1). Additionally, adjusting the distance between the second flat-fielding MLA and the Fourier lens fine tunes the periodicity of the excitation spots (Fig. 1e). This feature, not shared by refractive beam-shapers, allows us to optimize transmission efficiency.

Finally, in confocal microscopy and its variants such as iSIM, maximizing the achievable resolution requires focusing the excitation light to a diffraction-limited spot on the sample. However, implementing a traditional Köhler integrator in the excitation path produces large excitation spots due to the partially coherent extended source created by the rotating diffuser (Fig. 1e,f). In that case, the accessible improvement in resolution would not be based on diffraction-limited performance. A possible solution would be to introduce a pinhole array to mask the excitation spots, but at the expense of transmission efficiency.

In an alternative design, we find that—contrary to the typical Köhler integrator where illuminating a maximal number of flat-fielding microlenses is preferred\(^2\)\(^\text{2}\)\(^\text{2}\)\(^\text{2}\)\(^\text{2}\)\(^\text{2}\)—illuminating fewer microlenses offers a solution to ensure diffraction-limited excitation at the sample (Fig. 1g,h). Incorporating a beam de-expander to contract the light incident on the flat-fielding MLAs (Fig. 1i and Extended Data Fig. 1) allows us to tune the apparent size and angular distribution of the extended source. This shrinks the size of the excitation spots, while maintaining efficient light transmission and homogeneity. This configuration, which we call multifocal flat illumination for field-independent imaging (mFiFI), meets all the above requirements for homogeneous multifocal excitation and is shown in Fig. 1i.

These requirements can be described by geometrical optics and are a direct outcome of ray transfer matrix calculations (Supplementary Information and Supplementary Fig. 1), but the large parameter space makes the design and optimization of the mFiFI module nontrivial. For example, beam contraction presents a trade-off between homogeneity and spot size of the multifocal excitation and requires careful optimization (Extended Data Figs. 2 and 3). To facilitate the optimization and choice of components for efficient mFiFI, we provide the main design equations and an extended version of our existing wave optics simulation platform\(^9\) (Extended Data Figs. 2 and 3 and Supplementary Table 1).

iSIM integration and performance characterization. We tested the performance of the mFiFI module when integrated into the excitation path of an iSIM\(^8\)\(^\text{8}\)\(^\text{8}\)\(^\text{8}\) (Extended Data Fig. 4). To visualize the excitation illumination, we used a concentrated dye solution as a sample\(^2\)\(^\text{2}\)\(^\text{2}\)\(^\text{2}\), and imaged the emitted light onto the camera without scanning. Using an approximately Gaussian input beam (\(M^2<1.1\), full-width at half maximum (FWHM) diameter \(\sim 12\mu m\)) as in the initial iSIM design\(^8\)\(^\text{8}\), the spatially varying intensity of excitation points closely follows a Gaussian distribution (Fig. 2a), consistent with simulation (Fig. 1a). Scanning the excitation points partially homogenizes the excitation along the scan direction; however, the resulting illumination features a bright central region and strong roll-off away from the central optical axis (Supplementary Fig. 2). In comparison, generating the excitation using mFiFI results in a more uniform multifocal excitation covering an area larger than \(100 \times 100\mu m^2\) (Fig. 2b), and a more homogeneous excitation during scanning (Supplementary Fig. 2).

Comparing intensity profiles along the rows of excitation spots shows that mFiFI efficiently redistributes the input Gaussian excitation over a larger area (Fig. 2c). Quantifying these differences, we find that mFiFI doubles the number of excitation spots above background levels compared to the Gaussian excitation, while resulting in a narrower distribution of spot amplitudes and background (Fig. 2d,e,g). Furthermore, this improvement comes at no measurable cost to the quality of the structured illumination, since both the size of the excitation spots (Fig. 2f) and their periodicity (222.3±0.3 \(\mu m\) (Gaussian) and 222.2±0.3 \(\mu m\) (mFiFI) (mean±s.d.)) are maintained, and compare well with the ground truth (222 \(\mu m\) excitation MLA pitch). This was further verified by imaging 100 nm fluorescent beads, showing comparable lateral resolution between mFiFI (214±5 \(\mu m\) (mean±s.d.) and Gaussian illumination (219±12 \(\mu m\)), based on FWHM measured on raw iSIM data before deconvolution, or \(\sim 140\mu m\) lateral and \(\sim 350\mu m\) axial resolution after deconvolution) (Supplementary Fig. 3).

Finally, since iSIM is a scanning technique, it is imperative to ensure illumination homogeneity during the scan. Thus, we examined the relationship between scrambling speed of the rotating diffuser and averaging of diffraction patterns, thereby defining the parameter range necessary to achieve illumination homogeneity (Extended Data Fig. 3, Supplementary Information and Supplementary Videos 1–3).

Large FOV iSIM imaging and FOV stitching. We used our custom-built iSIM setup as a platform to combine mFiFI excitation with a scientific complementary metal-oxide semiconductor (sCMOS) camera for detection, to enable large FOV volumetric imaging at doubled resolution. While existing commercial SIM setups are mostly limited to FOVs with a linear dimension of 30–60 \(\mu m\), our setup reaches \(>100 \times 100\mu m^2\), thus providing a roughly four- to tenfold increase in FOV area. We used mFiFI-iSIM to simultaneously image multiple mammalian cells within a single FOV at doubled resolution (Fig. 3a and Supplementary Video 4). Accessible imaging speeds are limited merely by the frame rate of the detector; thus, frame rates of up to 100 Hz as reported for iSIM\(^8\)\(^\text{8}\)\(^\text{8}\)\(^\text{8}\) are
Fig. 1 | Design and features of the mfFIFI module. a, b, Wave optics simulation with a Gaussian (a) or flat-top beam input (b) onto the excitation MLA, showing their propagation and output intensity of generated excitation spots. c, d, Positions of excitation spots overlaid on a conjugate pinhole array and their relative displacement for nontelecentric (c) and telecentric illumination of the excitation MLA (d). Data from separately acquired images of pinholes and excitation spots. e, An excitation spot’s pitch depends on the Fourier lens back focal plane offset from the second flat-fielding MLA. Dashed line represents zero offset and the pitch of the MLA. f, g, Images of excitation spots using a Köhler integrator (f) and mfFIFI (g). Scale bar, 100 µm. h, Spot size increases with the diameter of the beam incident on the flat-fielding MLAs (n = 304). Error bars represent s.e.m., dashed line shows a linear dependence. i, Design and implementation of multifocal flat illumination for field-independent imaging (mfFIFI).
preserved, since the increase in FOV is achieved by improved parallelization from the increased number of illumination spots. Thus, the increase in FOV translates directly into an increase in throughput.

The effective image size can be further extended by stitching together adjacent FOVs. Such an approach requires homogeneous illumination to allow seamless stitching (Fig. 3b). The speed of iSIM imaging combined with the large FOV enabled by mfFIFI allowed us to stitch together dual-color 3D stacks covering a 500 × 500 × 5 μm³ volume with a 5 × 5 grid of FOVs, each acquired within 5 s. With this approach, within 2 min, we could capture 3D images of more than 80 cells stained for the mitochondrial inner (TIM23) and outer (TOM20) membrane translocases, revealing coupled geometries of the two membranes (Fig. 3b,c). The current limiting factor for multi-FOV imaging is the speed of stage translation and synchronization between stage translation and the rest of the iSIM acquisition control. In contrast, without mfFIFI, the spatially varying intensity produced dark boundaries at borders between FOV (Fig. 3b and Supplementary Fig. 2).

For time-lapse imaging, we introduced an additional layer of parallelization by iterating the acquisition procedure over a grid of FOVs (Fig. 3d). By trading temporal resolution to gain imaging area, we can use the speed of iSIM imaging to rapidly cover multiple FOVs. For example, mitochondria are motile organelles that require fast, high-throughput imaging to capture their dynamics and heterogeneity. By iterating over a 2 × 2 grid of FOVs we could reliably track at a temporal resolution of 2 s over 270 mitochondria and their contacts with the endoplasmic reticulum (Supplementary Fig. 2), known to serve as hotspots for mitochondrial division34 (Fig. 3e and Supplementary Video 5). This illustrates how dynamic processes can benefit from mfFIFI to extend the throughput of iSIM without compromising spatial resolution.

High-throughput super-resolution imaging of expanded centrioles. A variant of ExM termed U-ExM16 enables preservation of the structure and molecular identity of multiprotein assemblies (particles). When combined with iSIM imaging, an expansion factor of roughly four results in an effective resolution of ∼140/4 = 35 nm laterally and ∼350/4 ≈ 90 nm axially after deconvolution. Compared with current state-of-the-art super-resolution microscopes with similar resolution performance, such as the HT-STORM15 capable of imaging a 100 × 100 μm² FOV in ∼5–10 min or a similar FOV with easySLM-STED29 in 60–80 min, the iSIM is capable of stitching together images of expanded samples to form an equivalent FOV within 2–5 s. This represents a 100–1,000-fold improvement in imaging throughput. Therefore, datasets of thousands of particles that would take weeks or months to acquire on a conventional STORM or STED microscope require only 1–2 h on the high-throughput iSIM.

As a proof-of-concept for iSIM/U-ExM, we set out to analyze centrioles, organelles found in most eukaryotic cells that seed the formation of the axoneme in cilia and the centrosome in animal cells. Centrioles have a characteristic ninefold radial symmetric arrangement of microtubule triplets toward the proximal end of the organelle, composed of complete A microtubules and incomplete B and C tubules30, with a transition to A and B microtubule doublets toward the centriole distal end31 (Fig. 4a). Centriolar tubulin is enriched in PTMs, including acetylation and polyglutamylation (PolyE)32,33. Acetylation is known to stabilize microtubules, increase their flexibility and protect them against mechanical aging34,35. Glutamylation has been postulated to likewise stabilize microtubules in the centriole and protect the organelle from pulling and pushing forces acting during mitosis32,34. However, the spatial organization of these PTMs, which could lend further insight into their roles, remains to be fully explored.

To analyze centrioles in human cells at a similar stage, we synchronized RPE-1 cells with thymidine to arrest them at the G1/S transition and treated them with the Plk4 inhibitor Centrinone to prevent formation of new centrioles37. After expansion and staining with antibodies against acetylated and polyglutamylated tubulin, we imaged >100 centrioles per hour in 3D in their cellular context, revealing the distribution of these PTMs. Analyzing over 400 individual particles extracted from these images (Extended Data Fig. 5) revealed that centriolar tubulin appears uniformly acetylated, while PolyE terminates ∼30 nm before the distal end (Fig. 4b and Extended Data Fig. 6). Moreover, the PolyE signal exhibits a larger diameter (Fig. 4b and Extended Data Fig. 6), consistent with acetylation occurring inside the hollow microtubule and PolyE on its outer surface34.

To further increase throughput, we sought to expand purified centrioles to capture dozens of pairs within a single FOV rather than just one per cell. We used centrioles purified from C. reinhardtii since the U-ExM protocol had been optimized for this species34. Similar to what we observed with human centrioles in the cellular context, we found here that acetylated tubulin colocalizes with the microtubule wall along the entire length of the organelle (Fig. 4c), in agreement with a previous report34. We also found that tubulin glutamylation (GT335) is concentrated in the central core region of C. reinhardtii centrioles (Fig. 4d), as previously reported35. Since GT335 recognizes glutamylation but has lower affinity for longer

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**Fig. 2 | Performance comparison between Gaussian and mfFIFI excitation.** a, b, Excitation points imaged onto a NaFICTC fluorescent dye sample with Gaussian (a) and mfFIFI excitation (b). Scale bar, 10 μm. c, Intensity profiles measured along lines in a, b, d, Number of excitation points with a signal-to-noise ratio (SNR) above 3 in Gaussian and mfFIFI excitation. mfFIFI increases the number of excitation points by a factor of two. e–g, Histograms of excitation point amplitudes (e), variance (f) and background levels (g) for Gaussian and mfFIFI excitation (n = 1,805 for Gaussian and n = 3,620 for mfFIFI). e represents standard deviation/root-mean-square width/variance.
chains, we expected the distribution of PolyE to partially or completely overlap with that of GT335. We found that PolyE covers a wider territory along the length axis than GT335 (Fig. 4g,h and Extended Data Figs. 6 and 7). This difference could, however, reflect lower GT335 antibody labeling efficiency, since saturating the signal reveals similar GT335/PolyE coverage (Supplementary Fig. 4).

The high-throughput offered by mfFIFI-iSIM allowed us to collect 3D images of thousands of purified expanded C. reinhardtii centrioles per hour (Extended Data Fig. 8 and Supplementary Table 1). Having obtained three- rather than two-dimensional images allowed us to use fewer particles to perform averaging and high-resolution multicolor reconstruction compared to previous approaches using STORM or electron microscopy. Thus, we classified particles before reconstruction to identify and average only those particles sharing a high degree of similarity (ranging from 10 to 50% of the dataset), and thereby achieve even higher resolution reconstructions (Fig. 4f–h and Extended Data Fig. 9). Here we focused on the most frequently appearing class, but other classes could also be independently reconstructed. For mapping multiple PTMs within the centriole, we employ dual-color labeling, using α-tubulin staining of the microtubule triplet wall as a reference to subsequently align all PTMs (Extended Data Fig. 7).

The resulting reconstructions revealed an unexpected shift in the tangential position of the PolyE signal from the proximal to the distal end (Fig. 4h), also sometimes visible in individual raw particles (Extended Data Fig. 8 and Fig. 4e). To account for this shift, we considered two possibilities: (1) the whole microtubule triplet twists around its axis while the PolyE signal remains localized to the C microtubule giving it an apparent twist, and (2) the PolyE signal changes microtubule localization within the microtubule triplet. Arguing against the first possibility, the microtubule triplet twists only within ~100 nm of the proximal end, while in our reconstruction the PTM twist occurs over a wider region covering ~250 nm from the proximal end. Moreover, we determined that the first possibility should cause a moderate ~6 nm shift of the PolyE signal, and the second a more pronounced ~35–40-nm shift (Supplementary Information and Extended Data Fig. 10). From our reconstruction, we considered three evenly spaced axial locations while avoiding the extreme ends, and measure a twist of ~34.9 nm. This cannot be explained purely by twisting of the microtubule triplet (Fig. 4i) and Extended Data Fig. 10). Therefore, we propose that PolyE changes position within the microtubule triplet along the centriole, moving from the A microtubule to the C microtubule more distally.

Conclusion

We designed a flat-fielding method for efficient multifocal excitation, accompanied by a wave optics simulator that ensures optimal resolution performance. While the existing Köhler integrator has spot generating properties when used with coherent illumination, it introduces a strong wavelength dependence of the periodicity, making it unsuitable for multicolor applications. mfFIFI provides a largely wavelength-independent solution for generating homogeneous multifocal excitation, while benefiting from the homogenizing properties of the Köhler integrator. We implemented
the mfFIFI module into an iSIM, since its fast acquisition speeds and resolution doubling give it good potential for high-throughput super-resolution imaging. However, the mfFIFI module could be extended to any multifocal excitation microscope such as a spinning disk confocal microscope using a Nipkow disk, unlike other flat-fielding solutions using diffractive optical elements, SLMs or beam splitters. Furthermore, combining mfFIFI with phase masks required for donut beam shaping could extend its application to parallelized STED and reversible saturable optical fluorescence transition microscopes.

Fig. 4 | High-throughput super-resolution imaging of expanded centrioles for mapping PTMs of centriolar tubulin. a, Schematic representation of the centriolar microtubule wall viewed from the side (left) and top from the distal end. A, B and C microtubules are indicated. b, Examples of individual mature human centriole particles orthogonal (side view) or parallel (top view) to the optical axis collected in expanded RPE-1 cells labeled for acetylated tubulin and polyglutamylation (PolyE). The lateral (xy) maximum intensity projection and axial (xz) cross sections are shown. Scale bar, 100 nm. c–e, Examples of individual purified C. reinhardtii centriole particles viewed orthogonal (side view) or parallel (top view) to the optical axis labeled for α-tubulin as a reference and acetylated tubulin (c) (n = 2,129), glutamylated tubulin (d) (GT335, glutamylation, n = 6,527) and polyglutamylation tubulin (e) (PolyE, n = 1,865). f–h, High-resolution particle averaging reconstructions of acetylated tubulin (f) (n = 387), glutamylation (g) (n = 830) and PolyE (h) (n = 910) with α-tubulin as a reference. Scale bar, 100 nm (pre-expansion). Top views shown from the distal end. i, Circular axial projection (xz profile) of the centriole reconstruction from h showing α-tubulin and PolyE signal around the centriole (viewed from the outside). Scale bar, 100 nm. j, Intensity profiles measured along the dashed lines from i showing the tangential displacement of the PolyE signal.
Flat-fielding enables multi-FOV imaging, which is a powerful way to maintain resolution while increasing the effective image size. This is important for samples spanning multiple FOVs, such as tissues, or expanded samples. ExM sacrifices the effective FOV to increase resolution, and stitching provides a way to maintain throughput, allowing cellular features or structures to be imaged in situ, as we demonstrate by imaging hundreds of human centrioles in cells. While a similar effective resolution could be achieved on traditional diffraction-limited microscopes combined with a larger expansion factor, advantages to our approach include simpler expansion protocols and less sacrifice in throughput, providing the best of both worlds (Supplementary Information).

Particle averaging and reconstruction is traditionally used in ultrastructural biology, and leverages large datasets to capture the intrinsic variability within particles through classification before reconstruction, while averaging out the noise. In the case of ExM, different gels can have slightly different expansion factors, but this issue is circumvented by our iSIM setup since thousands of particles can be acquired from a single gel section (~5 × 5 mm²). In our case, the collected datasets were sufficiently large to allow particle classification before averaging, an approach that improves the resolution of the reconstruction. In addition to a 100–1,000-fold improvement in throughput of iSIM/U-ExM over HT-STORM 21,40 or easySLM-STED 29, expanded samples offer enhanced accessibility for antibodies to bind proteins in crowded assemblies. Beyond particle averaging, high-content or machine learning approaches rely on large datasets for training or screening and can benefit from the combination of throughput and resolution described here.

Our previous attempts to image centriolar microtubules with STORM proved largely unsuccessful, restricting previous studies to other epitopes 36. Here we reveal previously unobserved molecular organization, showing high coverage of PolyE in human centrioles, as well as precise localization to microtubule triplets along the C. reinhardtii centriole. We find that PolyE appears to shift from the A microtubule proximally to the C microtubule more distally; it is possible that such a glutamylation shift reflects a centriolar tube code that defines the spatial boundaries of the various elements that compose it, which could determine the positioning of different structural elements along the length of the organelle.

**Online content**

Any methods, additional references, Nature Research reporting summaries, source data, extended data, supplementary information, acknowledgements, peer review information; details of author contributions and competing interests; and statements of data and code availability are available at https://doi.org/10.1038/s41592-020-0859-z.

Received: 20 December 2019; Accepted: 13 May 2020; Published online: 22 June 2020

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Methods

Statistics. The $N$ values, which represent the number of data-points analyzed, are stated in figure legends and in the Methods section. Figures (Figs. 1–3 and Supplementary Fig. 2) show representative data from ≥3 experiments, or from six to twelve high-throughput experiments (Extended Data Figs. 5 and 8 and Supplementary Figs. 2 and 3). Extended Data Figs. 2–4 show representative data from three similar independent simulations. Multiple cutouts were imaged separately from individual gels. For different labelings, three gels were produced for PolyE, three for MonoE, one for acetylation for purified Chlamydomonas centrioles and two for expanded human RPE-1 cells, to ensure that the staining results were in general reproducible.

Sample preparation. Cos-7 and RPE-1 cells were grown in Dulbecco's modified eagle medium (DMEM) supplemented with 10% fetal bovine serum. Cells were plated on 25-mm, no. 1.5 glass coverslips (Menzel) 16–24 h before transfection or fixation at a confluency of ~10⁵ cells per well. Transfections using mito-GFP and KDEL-RFP were performed with Lipofectamine 2000 (Life Technologies) using 150 ng of mito-GFP and KDEL-RFP and 1.5 μl of the Lipofectamine 2000 reagent in 100 μl of Opti-MEM medium per six well.

For synchronization of RPE-1 cells at the G1/S transition and the onset of centriole assembly, ~125,000 cells were seeded for 24 h in six-well plates (Merck, T90206) on 12 mm coverslips in DMEM. Thymidine (Merck, T1895, 1 mM) and Centrinine (Lucerna-Chem, MCE-HY-18682, 300 mM) were added for 18 h, before fixation with cells with ~20°C methanol for 5 min.

For immunohistochemistry experiments with Cos-7 cells, cells were washed in prewarmed PBS before being fixed in prewarmed fixativebuffer (4% paraformaldehyde in PBS). Cells were then permeabilized by 0.5% Triton X-100 for 5 min. After washing in PBS for 5 min, cells were incubated in blocking buffer (1% BSA in PBS) for 30 min. The primary antibodies (Tom20-200 (1:150, FL-145 sc-11415 Santa Cruz Biotechnologies), Timm23-mouse (1:100, 611222 BD Biosciences)) diluted in blocking buffer were incubated for 60 min before washing three times in 0.2% BSA with 0.25% Triton-X in PBS for 5 min. Secondary antibodies (Alexa Fluor 488 goat antinouse IgG (H+L) (1:50, A28175 Thermofisher), Alexa Fluor 568 goat antirabbit IgG (H+L) (1:150, A10110 ThermoFisher)) were diluted in blocking buffer before incubation for another 30 min. The sample was incubated in the dark, then washed three times with PBS before imaging.

iSIM imaging. The iSIM setup was partly based on previously described implementations. Two lasers with wavelengths of 488 nm (Sapphire 488–300 CW CDRH, Coherent) and 561 nm (gem 561, Laser Quantum) were combined using a dichroic mirror (548–486-466, AHF Analysentechnik) and controlled through an acousto-optic tunable filter (AOTFInc–400–650–TN, AA Opto-electronic). In the case of Gaussian excitation, the beam was expanded with an 10x beam expander ($f_0 = 40 $ mm, $f_2 = 400 $ mm, Thorlabs). In the mFIFI path, a focusing lens ($f_0 = 50 $ mm) was used to focus the light near a rotating diffuser (2.5° × 0.25° FWHM at 650 nm, 24–09066, Süss MicroOptics) before collimation by a collimating lens ($f_0 = 50 $ mm, Thorlabs). The collimated beam was contracted by a factor of four by two lenses ($f_0 = 120 $ mm, $f_2 = 300 $ mm, Thorlabs) before illuminating two flat-fielding MLA (300 μm pitch, 10 × 10 mm², f=4.78 mm, square lenses, 18-00157, Süss MicroOptics), Fourier lens ($f_0 = 300 $ mm, Thorlabs). An iris (ID25, Thorlabs) is used before the two MLA to control the portion of the beam, which is allowed to propagate forward to reach and illuminate MLA, by adjusting the opening of the iris. An excitation MLA (300 μm pitch, 10 × 10 mm², f=4.78 mm, square lenses, 18-00157, Süss MicroOptics) is used to produce the multifocal points. The size of the multifocal illumination is reduced by a factor of 0.6x by a beam de-expander ($f_0 = 50 $ mm, f=30 $ mm, Thorlabs) to be of appropriate size for the sensor of the camera (DCC1545M, Thorlabs).

To obtain the data shown in Fig. 1e,f, we used the characterization platform to test the parameters affecting the spot size and pitch. A Vernier caliper was used to measure the iris opening diameter, which was varied methodically to control the size of the beam that illuminates the MLA. Images of the beam at different iris openings were analyzed using a MATLAB script, which allowed us to fit each excitation point to a two-dimensional Gaussian and measure the multifocal points using the FWHM of the Gaussian distribution. To study the variance of the pitch of the multifocal points, the distance between the Fourier lens and the second flat-fielding MLA was varied by displacing the flat-fielding MLA. A MATLAB script is used to measure the mean pitch at each distance, so that the relationship between this distance and the pitch can be determined.

Image analysis. Plateau uniformity quantification. The homogeneity was quantified using the plateau uniformity definition based on the FWHM of the histogram of the intensity values, according to ISO, ISO 13694:2018: Optics and optical instruments—lasers and laser-related equipment—test methods for laser beam power (energy) density distribution.

Illumination profile measurement. The illumination profiles used to optimize the pitch (teledendricty) and spot size of the multifocal excitation were visualized using a small color CMOS camera (DCC1645C, Thorlabs), placed in an intermediate image plane of the iSIM, between the second scan lens and tube lens, to avoid introducing other aberrations along the whole optical path of the iSIM. The excitation profile at the sample was measured using a highly concentrated fluorescent dye sample, as previously described. Briefly, sodium fluorescence (NaFITC) powder was diluted in deionized water, followed by vortexing and sonication until the powder was completely dissolved. A 10-μl drop was placed on a 25-mm coverslip, before covering with a 12-mm coverslip. The sample was then sealed using nail polish. The same dye sample was used to measure both the 488- and 561-nm illumination profiles. The illumination profiles were characterized by fitting a two-dimensional Gaussian to the intensity image. The threshold was set so that no points in the background were detected. The scanning profiles were measured with a 150-pixel-thick line in Fiji. In the case of excitation spots visualized in Fig. 1e,f, the illumination profile was imaged directly onto a camera placed in a conjugate image plane.

Bead FWHM analysis. To characterize the performance of the iSIM microscope, we used 100 nm Tetraspeck beads (ThermoFisher Scientific, T7279) deposited on poly-l-lysine treated coverslips. Bead size was determined by fitting a Gaussian profile to the intensity profile of the bead and extracting the FWHM.

Centriole shape and coverage analysis. To analyze the diameters of different PTM localizations within the centrioles, we measured the intensity profile through the centers of top view centrioles (central plane, centriole barrel parallel to the
Table 1. The propagation of an optical wave. An initial simulation showing the effect of optics algorithms and rely on angular spectrum propagation methods to simulate threshold, 2.50 for maximum/average displacement threshold and 3.50 for absolute centrioles). A 10-pixel-thick line and from summed intensity projections of side view centrioles coverage, we measured the intensity profile along the length of the centriole using diameter (which passed through the center axis) as the distance between the two peaks on opposing sides of the centriole ring.

To analyze the PTM distribution along the length of the centriole and their coverage, we measured the intensity profile along the length of the centriole using a 10-pixel-thick line and from summed intensity projections of side view centrioles (centriole barrel orthogonal to the imaging axis). We then took the width of the profile at one-quarter of the maximal signal as the length, to be less susceptible to noise. The coverage was then calculated by dividing the length of the PTM in question, by the length of the reference label (acetylated tubulin for human centrioles and α-tubulin in the case of C. reinhardtii centrioles).

Mitochondrial tracking. Mitochondria were segmented using Trainable Weka Segmentation and tracked by matching the closest neighboring center of mass positions between individual frames, as previously described.

Multiple FOV stitching. For stitching of multiple images, raw images or z stacks were acquired with a 10% overlap for both Gaussian and mfFIFI excitation. The acquired images were then combined using the Grad/Collection stitching tool in Fiji using the linear blending method with the default values of 0.30 for regression threshold, 2.50 for maximum/average displacement threshold and 3.50 for absolute displacement threshold.

To better quantify the variation in edge intensity in stitched images, we required a rather arbitrary threshold to be applied even if the whole image could be reliably stitched together. We used a dye lake sample (sodium fluorescein, NaFRTC), which had been left out for a few days and had time to crystallize, forming certain features that allowed it to be stitched while providing a sample of relatively uniform intensity.

We collected 3 × 3 grids of FOVs using both mfFIFI and Gaussian illumination, repeating the stitching for different stage displacements before aligning the resulting images (resulting in different degrees of overlap). We then stitched together the different 3 × 3 grids for mfFIFI and Gaussian illumination using the same parameters as above but using approximate stage coordinates instead of computing the overlap since this gave more reliable performance. After stitching the grid images for different stage displacements, before plotting the average vertical profile across the stitched image. The vertical profile is measured, since the horizontal scanning improves the illumination homogeneity along that direction. We then compared the minimal intensity measured at the edge to the mean intensity of the whole image. The ratio of the two is what we report as the ‘edge homogeneity’.

Wave optics simulations. The mfFIFI simulation platform has been adapted based on previous work. All simulations are based on standard numerical Fourier optics algorithms and rely on angular spectrum propagation methods to simulate the propagation of an optical wave. An initial simulation showing the effect of illuminating the excitation MLA with Gaussian and flat-fielded profiles was performed using phase masks to represent optical components.

The parameters used for the simulation are summarized in Supplementary Table 1.

Centriole expansion protocol. C. reinhardtii centrioles were isolated from the cell-wall-less strain CW15 (and expanded using the U-ExM protocol). Briefly, isolated centrioles were spun on 12 mm poly-d-lysine-coated coverslips before U-ExM. For human cell expansion, cells were initially seeded on 12 mm coverslips before fixation of cells with −20°C methanol for 5 min. Coverslips were then incubated in a 0.7% formaldehyde and 1% acrylamide in PBS for 4–5 h at 37°C. Next, coverslips were incubated in the monomer solution for 1 min on ice and then shifted to 37°C for 1 h in a dark and humidified chamber. For one gel, the monomer solution is made of 25 µl sodium acetate (Sigma-Aldrich, 405820, 38% wt/wt, diluted with nuclease-free water), 12.5 µl acrylamide (Sigma-Aldrich, A4058, 40% stock solution), 2.5 µl N,N′-methylenebisacrylamide (Sigma-Aldrich, M1533, 2% stock solution) and 5 µl 10X PBS, supplemented with 2.5 µl TEMED and 2.5 µl APS (from Sigma-Aldrich). Once polymerized, gels were moved to a destaining buffer (200 mM SDS, 200 mM NaCl and 50 mM Tris in nuclease-free water, pH 9) for 15 min at room temperature with gentle shaking and then shifted for 30 min to 95°C in an 1.5 ml Eppendorf tube with 1 ml of fresh denaturation buffer. Then, gels were placed at room temperature in beakers with 200 ml of distilled water. Water was exchanged twice (every 30 min) and the sample was incubated overnight at room temperature. The following day, water was changed with PBS for 15 min at room temperature. Gels were then incubated for 3 h at 37°C with gentle shaking in 1 ml of primary antibody solution (in 3% BSA and 0.05% Tween20 for human and 2% BSA in PBS for purified C. reinhardtii). Samples were then washed three times for 10 min with PBS supplemented with 0.1% Tween20 (PBS-T) while shaking, followed by a 15 min wash with secondary antibody solution. Finally, gels were washed three times in PBS-T. For RPE-1 cells, the sample was supplemented in the second wash with Hoechst 33258 (1:2000) dye and place in beakers with 200 ml of distilled water for final expansion, with again water being exchanged twice for 30 s. Before imaging, gels were again expanded overnight in ddH2O. Primary antibodies used to image centrioles in expanded RPE-1 cells were rabbit anti-polylactate chain (polyE), pAb (IN105, 1250) and mouse monoclonal anti-acetylated tubulin (Merck, T7431, 1:500). Secondary antibodies were goat antirabbit Alexa488 (ThermoFisher Scientific, A11008, 1:500) and goat antianti-α-tubulin (Lyse4,1:50, 32–2700, Invitrogen, ThermoFisher). Secondary antibodies were goat antirabbit Alexa Fluor 488 IgG H+L (1:400, A11008), goat antianti-α-tubulin (Lyse4,1:50, 32–2700, Invitrogen, ThermoFisher).

The final expansion factor was calculated by measuring the diameter of the expanded gel using a caliper and dividing the measured value by the original diameter of the coverslip.

Particle segmentation, classification and 3D reconstruction of purified centrioles. Particles were segmented using a custom written MATLAB script. Briefly, the threshold value was found on the maximum intensity projections of the 3D stacks, before applying it back to the individual stacks. The regions were then binarized and segmented by connecting neighboring pixels across the 3D stack. These particles were then filtered using a series of defined criteria that allowed them to be included in the final set.

After particles were segmented and up-sampled (to reach isotropic pixel size), the particles with tubulin signal were aligned to a reference using Dynamo. The reference was built using a reconstructed tubulin signal made from 12 manually selected particles. Once particles were all similarly aligned, a cross-correlation matrix was generated by calculating the similarity between each pair of particles. The cross-correlation matrix was converted to a distance matrix by subtracting cross-correlation values to one. A hierarchical classification was made using the Ward method (hclust function from R). The classification tree was empirically cut into ten groups. For each group, the average particle was generated for the tubulin signal and for the polyE signal by applying the transformation parameters obtained during the alignment step. The ten averages were compared to identify which groups were most promising. The best classes were used to produce the reconstruction results of Fig. 4. The reconstruction method was based on ref. 35, and followed the procedure reported in ref. 34 with a C9 symmetry constraint. The point spread function was experimentally measured by imaging 100 nm fluorescent beads (TetraSpeck Microspheres, 0.1 µm, T7279 ThermoFisher). We made two modifications to the reference-free step of the reconstruction algorithm. First, we adopted a multiscale reconstruction approach: we used results obtained with coarsely subsampled data to get a coarse initialization. Thanks to this approach, we were able to perform the reconstruction at the highest resolution in reasonable computation time. Second, we decoupled the angular search of the orientations: the first two Euler angles were estimated to begin with, before the third one. This further accelerated the reconstruction and provided sharper results in the case of C9 symmetry.

Reconstructions were visualized using Chimera, by setting the threshold to fit the average length of the particles. Artifactual signals in the center of the reconstruction that arise due to the imposed ninefold symmetry were removed during postprocessing.

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

Data availability. The datasets generated and analyzed in this study are available from the corresponding authors upon request. Sample datasets have been made available on Zenodo (https://zenodo.org/record/361906#Xjib51NKKTF).

Code availability. The wave optics simulation platform for optimizing mfFIFI is provided as Supplementary Software 1. Updated versions can be found on GitHub (https://github.com/dmaletic/simmla_w_mfFIFI). The custom MATLAB source code for aligning 3D particles is available at https://github.com/dfortun2/U-ExM'.

References. 44. Edelstein, A. D. et al. Advanced methods of microscope control using µManager software. J. Biol. Methods 1, 10 (2014).

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We thank H. Shroff and A. Curd for their help and advice on the construction of the iSIM; H. Perreten for technical assistance; C. Berz for her help with multi-FOV imaging; R. Kirchner on discussions on the Köhler integrator and C. Sieben and L. Carlini for critical reading of the manuscript. This work is supported by the European Research Council (grants AdG 835322, CENGIN to P. Gönczy and StG 715289 ACCENT to P. Guichard), the Swiss National Science Foundation (SNSF) grants PP00P3_157517 to P. Gu and 182429 to S.M., the MSCA (grant 75200, CARTASSY to N.B.), and the NCCR Chemical Biology (S.M. and P. Gönczy).

Author contributions
D.M., K.M.D., P. Gönczy, V.H., P. Guichard and S.M. conceived and designed the project. P. Gönczy, V.H., P. Guichard and S.M. supervised the project. D.M., K.M.D. and S.M. designed the multifocal illumination system. D.M. and K.M.D. developed the simulation platform. D.M. built the microscope, performed all experiments and data analysis. D.G. performed the purified Chlamydomonas reinhardtii centriole sample preparation. D.F. performed single particle averaging and reconstruction. N.B. performed the human RPE-1 centriole sample preparation. M.L.G. performed the particle classification and alignment. K.A.I. built and performed experiments on the flat-fielding characterization platform. D.M. and S.M. wrote the manuscript with contributions from all authors.

Competing interests
The mfFIFI flat-fielding module has been covered in a patent application filed by EPFL, with D. Mahecic, K.M. Douglass and S. Manley as the inventors and is currently pending under patent application number PCT/EP2018/085228.

Additional information
Extended data is available for this paper at https://doi.org/10.1038/s41592-020-0859-z.
Supplementary information is available for this paper at https://doi.org/10.1038/s41592-020-0859-z.
Correspondence and requests for materials should be addressed to D.M. or S.M.
Peer review information Rita Strack was the primary editor on this article and managed its editorial process and peer review in collaboration with the rest of the editorial team.
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Extended Data Fig. 1 | mFIFI development stages. a) A standard Köhler uses a pair of flat-fielding MLAs to split the incoming light into multiple channels, before focusing them in the front-focal plane of the Fourier lens. In the case of a coherent light source, such as lasers, a focusing lens and a rotating diffuser are used to scramble the incoming light and generate an extended partially coherent source, which is then collimated by the collimating lens. Implementing a traditional Köhler integrator with a variable length $L_3$ between the second flat-fielding MLA and the Fourier lens, results in nontelecentric illumination of the excitation MLA. This will in turn cause the pitch of the excitation spots produced by the excitation MLA to vary. b) The telecentric Köhler integrator assures that the pitch of the excitation spots generated by the excitation MLA is conserved, by setting the distance between the second flat-fielding MLA and the Fourier lens to the focal length of the Fourier lens: $L_3 = f_\text{FL}$. The partially coherent extended source will however limit the capability of the microscope to achieve diffraction-limited excitation at the sample. A possible solution would be to limit the size of the spots by placing a pinhole array in the front focal plane of the excitation MLA, although at a cost to the transmission efficiency. c) The extended design overcomes these problems by introducing a beam expander between the collimating lens and the first flat-fielding MLA, which allows control over how many microlens channels are used to average over in the focus of the Fourier lens. Alternatively, placing a hard aperture to limit the radius of the beam incident on the flat-fielding MLAs would have a similar effect, albeit rejecting much of the incident light.
Extended Data Fig. 2 | Optimization of design parameters using the extended simulation platform. 

a) Schematic showing the standard Köhler integrator setup, indicating how the different design parameters are defined in the simulation.  
b) Field amplitude at the front focal plane of the Fourier lens, corresponding to the field incident on the excitation MLA, and c) at the front focal plane of the excitation MLA for different distances $L_2$ of the Fourier lens from the flat-fielding MLAs. The telecentric condition corresponds to $L_2 = 0$.  
d) Average pitch of the multifocal excitation measured at the front focal plane of the excitation MLA for different distances $L_2$. Dashed line marks the actual pitch of the excitation MLA.  
e) Schematic representation showing the mfFIFI configuration including the beam contractor and labeling the simulation parameters. The beam contraction factor is set by the inverse of the magnification of the two lenses of the beam contractor: $F_2/F_1$.  
f) Field amplitude at the front focal plane of the Fourier lens and g) the front focal plane of the excitation MLA.  
h) Trade-off of the beam contraction factor between the spot size (left axis, $n = 87$ excitation peaks) and the homogeneity of the excitation spots (right axis), quantified by the plateau uniformity; the central mark on the boxplot indicates the median, and the bottom and top edges of the box indicate the 25th and 75th percentiles, respectively. Shown data representative out of $>3$ similar independent simulations.
Extended Data Fig. 3 | Quantifying excitation homogeneity as function of the scrambling speed of the rotating diffuser. a) Representative simulated data showing the relative field intensity in the plane of the excitation MLA and b) at the front focal plane of the excitation MLA. c–d) Variation in excitation spot localization, width and amplitude for illumination averaged over different numbers of simulated random wavefronts (N = 909 binned peak variations). f) Real data showing the field intensity in the plane of the excitation MLA and g) at the front focal plane of the excitation MLA. In this case the two fields were not acquired at the same random wavefront, but are intended to give a general idea of what the fields look like. h–j) Variation in excitation spot localization, width and amplitude for illumination averaged over different numbers of random wavefronts acquired experimentally (N = 4248 binned peak variations). k–m) Dependence of the spot localization, width and amplitude as function of the number of iterations over which the illumination is averaged over (representing different scrambling speeds, or acquisition speeds). n–p) Variation in the localization, width and amplitude between individual random wavefronts for simulated (N = 499900 individual peak variations) and experimentally acquired data (N = 973736 individual peak variations). Shown simulated data representative out of >3 similar independent simulations.
Extended Data Fig. 4 | Integrating mfFIFI into an instant structured illumination microscope. **a)** Schematic representation of the iSIM setup, showing where the mfFIFI module is integrated into the excitation path prior to the excitation MLA. **b)** Simulated flat-field incident on the excitation MLA. **c)** Simulated intensity profile in the front-focal plane of the excitation MLA, showing an array of excitation points (inset). Simulated data representative out of >3 similar independent simulations.
Extended Data Fig. 5 | Centriole particles from expanded RPE-1 cells. Montage of a random subset of unclassified raw particles collected in situ from expanded synchronized human RPE-1 cells stained for acetylated tubulin (yellow) and PolyE (magenta). Scale bar: 1µm.
Extended Data Fig. 6 | Particle shape analysis and PTM coverage. a) Particle lengths measured along side views of expanded human centrioles in the cellular context. b) Particle diameters measured on top views of human centrioles. c) PolyE coverage for human centrioles with respect to the acetylated tubulin signal along the length of the centriole. d) PTM length coverage measured along side views of purified *Chlamydomonas reinhardtii* centrioles. e) Particle diameters measured on top views of purified *Chlamydomonas reinhardtii* centrioles. f) PTM coverage for purified *Chlamydomonas reinhardtii* centrioles measured by dividing the length profiles of different PTMs by their respective α-tubulin signal. All scales reflect pre-expansion size. N = 25 top views and N = 41 side views for human centrioles. N = 50 for acetylation dataset, N = 49 for GT335 dataset and N = 50 for PolyE dataset in *Chlamydomonas reinhardtii*. The center line represents the mean and error bars represent the standard deviation.
Extended Data Fig. 7 | Multi-color particle averaging and reconstruction of tubulin PTMs in Chlamydomonas reinhardtii centrioles. a) Side and top views of α-tubulin reference with different PTMs. Top views are taken from the distal side toward the proximal side. Scale bar: 100 nm.
Extended Data Fig. 8 | Expanded purified Chlamydomonas reinhardtii centrioles. a–c) Montages of maximum intensity projections of a subset of centriole particles collected with dual-staining for a) acetylated tubulin, b) PolyE and c) GT335 with α-tubulin used as reference in each case. Inset in b) shows examples of PolyE twisting as single color maximum intensity projections. Scale bars: 1µm.
Extended Data Fig. 9 | Particle classification prior to reconstruction. Dendrograms generated via hierarchical classification in 10 groups. On top, the average volume of all particles is displayed with 2 different orientations. For each group, the average volume is shown with the same 2 orientations, the tubulin signal dark-coloured and the (a, b) acetylated, (c, d) glutamylated and (e, f) polyglutamylated signal light-coloured.
Extended Data Fig. 10 | Twist of polyglutamylated tubulin along *Chlamydomonas reinhardtii* centriole. a) Schematic illustration of centriolar microtubule triplets and expected radial and tangential displacements between microtubules in neighbouring triplets (inset) viewed from the proximal end. b) Schematic representation of XY planes between the shift and twist models of PolyE shift along the proximal-distal centriole axis and their predictions (cross sections viewed from the proximal end). c) Cross section of the reconstruction YZ profile showing the barrel diameter with α-tubulin and PolyE signal. d) Radial position of PolyE signal relative to α-tubulin, measured at different positions along the reconstructed centriole for all 9 microtubule triplets, from the proximal to the distal end. Points mark the mean of the N = 9 triplet measurements and error bars their standard deviation. e) The relative radial PolyE shift was calculated with respect to the α-tubulin signal by taking the difference between their radial positions, determined by Gaussian fitting. The profiles were measured from top views of the reconstructed centriole (inset). f, g) The relative tangential shift of PolyE is measured from the tangential projection (inset) with respect to α-tubulin at the f) distal and g) proximal end.
Reporting Summary

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Our web collection on statistics for biologists contains articles on many of the points above.

Software and code

Policy information about availability of computer code

Data collection

A custom written MATLAB code was used for iSIM acquisition control, version Matlab 2016b (version 9.1.0.441655) for Windows 10 (version 10.0.17763 Build 17763), in combination with the freely available software Micromanager 2.0 beta (version 2.0.0-beta3 20180905). All developed code will be made publicly available upon request.

Data analysis

Wave optics simulations were performed using a custom written Python code run using the Google colab interface. All other data analysis was developed, performed and tested in MATLAB 2016a (version 9.0.0.341360) for Windows 10 (version 10.0.17763 Build 17763). All developed code will be made available upon request.

Particle averaging and reconstruction was performed using custom written MATLAB code based on previously published work (Fortun et al., IEEE Trans. Med. Imaging 2018) and run using Matlab version 2016a (version 9.0.0.341360). The MATLAB source code is available at https://github.com/dfortun2/U-ExM. Particle classification was done using Dynamo (v-1.1.212). Reconstructions were visualized using Chimera version 1.13.1. Other data analysis was done using Fiji (ImageJ) version 1.52p.

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All manuscripts must include a data availability statement. This statement should provide the following information, where applicable:

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- A description of any restrictions on data availability

The datasets generated and analyzed in this study are available from the corresponding authors upon request. Sample datasets have been made available on
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Life sciences study design

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

Sample size
No hypothesis-based experiment was performed and therefore there was no predetermined sample size. Sample sizes were chosen based on how many images were required for 3D reconstruction and could be collected within 1-2 hours, which usually amounted to ~100 fields-of-view and 1000-10000 centriole particles. Each sample was analyzed separately. Note that we stated in the manuscript how many centrioles were analyzed in each experiment. In other imaging experiments, all representative data and experiments were repeated >3 times.

Data exclusions
Datasets with antibodies that stained in a manner inconsistent with the published literature was excluded from the analysis. These exclusion criteria were established based on published literature. Those experiments were repeated until results could be reproduced over multiple (>3) replicates. These repeatable results were consistent with previously published literature.

Replication
Multiple cut outs were imaged separately from individual gels. For different labelings, 3 gels were produced for PolyE, 3 for MonoE, 1 for acetylation for purified chlamydomonas centrioles and 2 for expanded human RPE-1 cells, to ensure that the staining results were in general reproducible. All replication attempts were successful except for a single MonoE gel where the staining was not consistent with previously published literature and previous experiments. We speculate that this is due to a mix-up between the secondary antibodies used.

Randomization
This is not relevant to our study. There was not a group allocation component to our study.

Blinding
There was not a group allocation component to the study.

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| [ ] | ChIP-seq              |
| [ ] | Flow cytometry        |
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Antibodies

Antibodies used
All antibodies used are listed in the methods section. For fixed cell imaging, we used Tom20-rabbit (1:50, FL-145 sc-11415 Santa Cruz Biotechnologies), and Tim23-mouse (1:100, 611222 BD Biosciences) as primary antibodies. Secondary antibodies were AlexaFluor 488 goat anti-mouse IgG (H+L) (1:150, A28175 ThermoFisher), AlexaFluor 568 goat anti-rabbit IgG (H+L) (1:150, A11011 ThermoFisher).

For expansion in human RPE-1 cells, primary antibodies were used were rabbit anti-polyglutamate chain (polyE), pAb (IN105, 1:250) and mouse monoclonal anti-acetylated tubulin (Merck, T7451, 1:500). Secondary antibodies were goat anti-Rabbit Alexa488 (Thermo-Fisher Scientific, A11034, 1:500) and goat anti-mouse Alexa Fluor 568 Fl(ab)2 (Thermo-Fisher Scientific, A11039, 1:500).

For expansion of purified Chlamydomonas reinhardtii centrioles, primary antibodies used were rabbit polyclonal anti-polyglutamate chain (PolyE, IN105, 1:500, AG-250-0030-C050, Adipogen), mouse monoclonal anti-α-tubulin (DM1a, 1:500, T6199, Sigma-Aldrich), mouse monoclonal antipolyglutamylation modification (GT335, 1:200, AG-208-0020, Adipogen), mouse monoclonal anti-acetyl-α-tubulin (Lys40, 1:50, 32-2700, Invitrogen, ThermoFisher). For expansion of purified Chlamydomonas reinhardtii centrioles, primary antibodies used were rabbit polyclonal anti-polyglutamate chain (PolyE, IN105, 1:500, AG-250-0030-C050, Adipogen), mouse monoclonal anti-α-tubulin (DM1a, 1:500, T6199, Sigma-Aldrich), mouse monoclonal antipolyglutamylation modification (GT335, 1:200, AG-208-0020, Adipogen), mouse monoclonal anti-acetyl-α-tubulin (Lys40, 1:50, 32-2700, Invitrogen, ThermoFisher). Secondary antibodies were goat anti-rabbit Alexa Fluor 488 IgG H+L (1:400, A11008), goat anti-mouse Alexa Fluor 488 IgG H+L (1:400, A11029), goat anti-rabbit Alexa Fluor 568 IgG H+L (1:400, A11036), goat anti-mouse Alexa Fluor 568 IgG H+L (1:400, A11004 all from Invitrogen, ThermoFisher).

Validation
Information regarding validation and application can be found in the manufacturers website:
- Rabbit monoclonal anti-Tom20 (FL-145, sc-11415): https://www.scbt.com/p/tom20-antibody-fl-145
- Purified mouse anti-Tim23 (611222): https://wwwbdbiosciences.com/us/applications/research/apoptosis/purified-antibodies/
Eukaryotic cell lines

Policy information about cell lines

| Cell line source(s)                      | RPE-1 cell line: purchased by PG from ATCC. 
|                                         | COS-7 cell line: purchased by SM from HPA Culture Collections. |
| Authentication                           | The cell lines used in this study were not authenticated by the authors. |
| Mycoplasma contamination                  | The used cell lines are routinely tested for mycoplasma contamination and cells are discarded if positive. |
| Commonly misidentified lines (See ICLAC register) | We did not use commonly misidentified cell lines. |