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 $>100 \times 100 \mu\text{m}^2$ 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 cells. 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 $\sim 35 \text{ nm}$. Classification and particle averaging of these large datasets enables 3D mapping of posttranslational modifications of centriolar microtubules, revealing differences in their coverage and positioning.

Super-resolution fluorescence techniques have enabled optical imaging beyond the diffraction limit. Two main illumination strategies are used to achieve super-resolution: wide-field illumination is typically used for single-molecule localization microscopies (SMLM) such as STORM¹ and PALM², while patterned illumination is typically used for methods such as structured illumination microscopy (SIM)³ and stimulated emission depletion (STED)⁴. Initial implementations of these methods were relatively slow, in part due to using the time domain to separate single molecules (SMLM), collecting multiple images to cover Fourier space (SIM), or scanning a reduced effective excitation volume (STED). In both wide-field and patterned illumination, the imaging throughput—defined as the area imaged per time—can be increased by parallelizing the acquisition, provided the necessary illumination can be maintained over a larger surface in the sample plane. Extending the array of patterned excitation has been used to increase the speed or FOV of confocal⁵, STED^{6,7} and SIM imaging^{8,9}, but ensuring the quality of the extended pattern across a larger FOV remains a limiting factor. For SMLM, flat-fielding approaches made it possible to extend super-resolution imaging over $\sim 100 \times 100 \mu\text{m}^2$ FOVs^{10,11}, but their transfer to patterned illumination remains limited.

An entirely different approach to effectively achieve super-resolution modifies sample preparation rather than image acquisition. Expansion microscopy¹² (ExM) physically increases the specimen size, isotropically, allowing higher imaging throughput by opting for faster diffraction-limited microscopes^{12,13} while the resolvable scale is reduced by the expansion factor. Combining ExM with existing super-resolution microscopies allows further improvement in resolution^{13–16}. However, expansion exacerbates limitations in imaging throughput since the effective FOV size is

divided by the expansion factor along each dimension¹⁷. Therefore, fast, parallelized, super-resolution techniques with large FOVs present an advantage for imaging ExM samples.

The iSIM⁸ uses parallelization in the form of multifocal excitation and optical image processing to achieve a two-fold improvement in resolution, at frame rates reaching 100 Hz. While this gives the iSIM good potential for high-throughput, spatially varying illumination produces a spatially dependent signal-to-noise ratio while typically restricting the imaging FOV to $\sim 50 \times 50 \mu\text{m}^2$. Although solutions for producing homogeneous multifocal excitation exist using diffractive optical elements¹⁸, spatial light modulators (SLM)¹⁹, multimode fibers²⁰ or beam splitters²¹, each suffers from some combination of a limited number of excitation spots^{18,19,21}, heterogeneity^{19,21} or low transmission efficiency¹⁸.

In this work, we develop a flat-fielding element for multifocal excitation, dubbed mfFIFI. Our design, based on the Köhler integrator^{22,23}, ensures that the homogeneity, pitch and size of the excitation spots are optimized. We integrate mfFIFI into an iSIM to engineer an instrument capable of 3D multicolor imaging with uniform quality and doubled resolution over a $100 \times 100 \mu\text{m}^2$ FOV. We further parallelize the acquisition across multiple FOVs, stitching them to create seamless montages of tens of super-resolved living cells within seconds, enabled by the homogeneous illumination. Finally, we combine the mfFIFI-iSIM with ultrastructure-ExM (U-ExM)²⁴ to perform high-throughput imaging with an effective resolution of $\sim 35 \text{ nm}$. We use this to collect 3D images of hundreds of expanded centrioles in human cells, and thousands of expanded purified *C. reinhardtii* centrioles. These particles are averaged to reconstruct maps of posttranslational modifications (PTMs) of centriolar tubulin, revealing differences in the spatial organization of acetylation, glutamylation and polyglutamylation.

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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^{22,23}, 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)^{22,23}.

Previously, multifocal excitation in iSIM was generated by illuminating a MLA with a collimated Gaussian beam^{5,8,9}. 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 second flat-fielding MLA²⁵, 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^{22,23}—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 (mfFIFI), 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 mfFIFI 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 mfFIFI, we provide the main design equations and an extended version of our existing wave optics simulation platform¹⁰ (Extended Data Figs. 2 and 3 and Supplementary Table 1).

iSIM integration and performance characterization. We tested the performance of the mfFIFI module when integrated into the excitation path of an iSIM^{8,26} (Extended Data Fig. 4). To visualize the excitation illumination, we used a concentrated dye solution as a sample²⁷, 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 ~ 12 mm) as in the initial iSIM design^{8,26}, 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 mfFIFI results in a more uniform multifocal excitation covering an area larger than $100 \times 100 \mu\text{m}^2$ (Fig. 2b), and a more homogeneous excitation during scanning (Supplementary Fig. 2).

Comparing intensity profiles along the rows of excitation spots shows that mfFIFI efficiently redistributes the input Gaussian excitation over a larger area (Fig. 2c). Quantifying these differences, we find that mfFIFI 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 ($222.3 \pm 0.3 \mu\text{m}$ (Gaussian) and $222.2 \pm 0.3 \mu\text{m}$ (mfFIFI) (mean $\pm$ s.d.)) are maintained, and compare well with the ground truth ($222 \mu\text{m}$ excitation MLA pitch). This was further verified by imaging 100 nm fluorescent beads, showing comparable lateral resolution between mfFIFI (214 ± 5 nm, mean $\pm$ s.d.) and Gaussian illumination (219 ± 12 nm), based on FWHM measured on raw iSIM data before deconvolution, or ~ 140 nm lateral and ~ 350 nm axial resolution after deconvolution⁸ (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 mfFIFI 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 – $60 \mu\text{m}$, our setup reaches $>100 \times 100 \mu\text{m}^2$, thus providing a roughly four-to-tenfold increase in FOV area. We used mfFIFI-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^{8,26} 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).

Fig. 2 | Performance comparison between Gaussian and mfFIFI excitation. **a, b**, Excitation points imaged onto a NaFITC 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). σ represents standard deviation/root-mean-square width/variance.

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 $500 \times 500 \times 5 \mu\text{m}^3$ 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 division²⁸ (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-ExM²⁴ 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 $\sim 140/4 = 35$ nm laterally and $\sim 350/4 \approx 90$ nm axially after deconvolution. Compared with current state-of-the-art super-resolution microscopes with similar resolution performance, such as the HT-STORM¹⁰ capable of imaging a $100 \times 100 \mu\text{m}^2$ 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 aging^{34,35}. Glutamylation has been postulated to likewise stabilize microtubules in the centriole and protect the organelle from pulling and pushing forces acting during mitosis^{33,36}. 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 surface²⁴.

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 species²⁴. 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 report³⁸. We also found that tubulin glutamylation (GT335) is concentrated in the central core region of *C. reinhardtii* centrioles (Fig. 4d), as previously reported³⁸. Since GT335 recognizes glutamylation but has lower affinity for longer

Fig. 3 | Large FOV imaging and multi-FOV stitching. **a**, Cos-7 cell expressing KDEL-RFP imaged with an iSIM using mfFIFI excitation and an sCMOS camera enabling imaging of FOVs up to $\sim 115 \times 115 \mu\text{m}^2$ at doubled resolution compared to diffraction-limited performance. The yellow square shows the size of a $30 \times 30 \mu\text{m}^2$ FOV available on a standard commercial SIM setup. Full video shown in Supplementary Video 4. Scale bar, $10 \mu\text{m}$. **b, c**, Raw iSIM images of Cos-7 cells immunolabeled for TIM23 and TOM20, stitched from a 5×5 grid of FOVs using Gaussian (**b**) and mfFIFI excitation (**c**). Scale bars, $50 \mu\text{m}$. **d**, Schematic of additional parallelization of image acquisition. **e**, Montage showing frames from a high-speed video built up over a 2×2 grid of FOVs (original video 2 s temporal resolution) of cells expressing mito-GFP and KDEL-RFP. Scale bars, $50 \mu\text{m}$ (upper) and $10 \mu\text{m}$ (inset). Full video shown in Supplementary Video 5.

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 2). Having obtained three- rather than two-dimensional images allowed us to use fewer particles to perform averaging and high-resolution multicolor reconstruction³⁹ compared to previous approaches using STORM⁴⁰ or electron microscopy⁴¹. 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 microtubule⁴² 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 $\sim 100 \text{ nm}$ of the proximal end⁴³, while in our reconstruction the PTM twist occurs over a wider region covering $\sim 250 \text{ nm}$ from the proximal end. Moreover, we determined that the first possibility should cause a moderate $\sim 6 \text{ nm}$ shift of the PolyE signal, and the second a more pronounced $\sim 35\text{--}40\text{-nm}$ 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 $\sim 34.9 \text{ nm}$. This cannot be explained purely by twisting of the microtubule triplet (Fig. 4i,j and Extended Data Fig. 10). Therefore, we propose that PolyE changes position within the microtubule triplet along the centriole, moving from the A microtubule on the proximal end 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 applications²³. mfFIFI provides a largely wavelength-independent solution for generating homogeneous multifocal excitation, while benefiting from the homogenizing properties of the Köhler integrator. We implemented

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 polyglutamylated 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.

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^{5,9} using a Nipkow disk, unlike other

flat-fielding solutions using diffractive optical elements¹⁸, SLMs¹⁹ or beam splitters²¹. 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.

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 ($\sim 5 \times 5 \text{ mm}^2$). 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^{10,40} or easySLM-STED²⁹, 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 epitopes⁴⁰. 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 tubule 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>.

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References

- Rust, M. J., Bates, M. & Zhuang, X. Sub-diffraction-limit imaging by stochastic optical reconstruction microscopy (STORM). *Nat. Methods* **3**, 793–796 (2006).
- Betzig, E. et al. Imaging intracellular fluorescent proteins at nanometer resolution. *Science* **313**, 1642–1645 (2006).
- Gustafsson, M. G. L. Surpassing the lateral resolution limit by a factor of two using structured illumination microscopy. *J. Microsc.* **198**, 82–87 (2000).
- Hell Stefan, W. & Jan, W. Breaking the diffraction resolution limit by stimulated emission: stimulated-emission-depletion fluorescence microscopy. *Opt. Lett.* **19**, 780–782 (1994).
- Tanaami, T. et al. High-speed 1-frame/ms scanning confocal microscope with a microlens and Nipkow disks. *Appl. Opt.* **41**, 4704 (2002).
- Bingen, P., Reuss, M., Engelhardt, J. & Hell, S. W. Parallelized STED fluorescence nanoscopy. *Opt. Express* **19**, 23716 (2011).
- Chmyrov, A. et al. Nanoscopy with more than 100,000 ‘doughnuts’. *Nat. Methods* **10**, 737–740 (2013).
- York, A. G. et al. Instant super-resolution imaging in live cells and embryos via analog image processing. *Nat. Methods* **10**, 1122–1126 (2013).
- Schulz, O. et al. Resolution doubling in fluorescence microscopy with confocal spinning-disk image scanning microscopy. *Proc. Natl Acad. Sci. USA* **110**, 21000–21005 (2013).
- Douglass, K. M., Sieben, C., Archetti, A., Lambert, A. & Manley, S. Super-resolution imaging of multiple cells by optimized flat-field epi-illumination. *Nat. Photonics* **10**, 705–708 (2016).
- Archetti, A. et al. Waveguide-PAINT offers an open platform for large field-of-view super-resolution imaging. *Nat. Commun.* **10**, 1267 (2019).
- Chen, F., Tillberg, P. W. & Boyden, E. S. Expansion microscopy. *Science* **347**, 543–548 (2015).
- Gao, R. et al. Cortical column and whole-brain imaging with molecular contrast and nanoscale resolution. *Science* **363**, eaau8302 (2019).
- Cahoon, C. K. et al. Superresolution expansion microscopy reveals the three-dimensional organization of the *Drosophila* synaptonemal complex. *Proc. Natl Acad. Sci. USA* **114**, 6857–6866 (2017).
- Gao, M. et al. Expansion stimulated emission depletion microscopy (ExSTED). *ACS Nano* **12**, 4178–4185 (2018).
- Xu, H. et al. Molecular organization of mammalian meiotic chromosome axis revealed by expansion STORM microscopy. *Proc. Natl Acad. Sci. USA* **116**, 18423–18428 (2019).
- Mahecic, D., Testa, I., Griffié, J. & Manley, S. Strategies for increasing the throughput of super-resolution microscopies. *Curr. Opin. Chem. Biol.* **51**, 84–91 (2019).
- Sacconi, L. et al. Multiphoton multifocal microscopy exploiting a diffractive optical element. *Opt. Lett.* **28**, 1918 (2003).
- Coelho, S. et al. Multifocal multiphoton microscopy with adaptive optical correction. *Multiphot. Microsc. Biomed. Sci.* **XIII** **8588**, 858817 (2013).
- Cooper, D. J. F. Imaging distal end of multimode fiber. US Patent 867,017,8B2 (2014).
- Nielsen, T., Fricke, M., Hellweg, D. & Andresen, P. High efficiency beam splitter for multifocal multiphoton microscopy. *J. Microsc.* **201**, 368–376 (2001).
- Zimmermann, M., Lindlein, N., Voelkel, R. & Weible, K. J. Microlens laser beam homogenizer: theory application. In *Proc. SPIE 6663, Laser Beam Shaping VIII* 666302 (SPIE, 2007).
- Voelkel, R. & Weible, K. J. Laser beam homogenizing: limitations and constraints. In *Proc. SPIE 7102, Optical Fabrication, Testing, and Metrology III* 71020J (eds. Duparré, A. & Geyl, R.) (2008).
- Gambarotto, D. et al. Imaging cellular ultrastructures using expansion microscopy (U-ExM). *Nat. Methods* **16**, 71–74 (2019).
- Schreiber, P., Kudaev, S., Dannberg, P. & Zeitner, U. D. Homogeneous LED-illumination using microlens arrays. *Nonimaging Opt. Effic. Illum. Syst.* **II** **5942**, 59420K (2005).
- Curd, A. et al. Construction of an instant structured illumination microscope. *Methods* **88**, 37–47 (2015).
- Model, M. A. & Blank, J. L. Concentrated dyes as a source of two-dimensional fluorescent field for characterization of a confocal microscope. *J. Microsc.* **229**, 12–16 (2008).
- Friedman, J. R. et al. ER tubules mark sites of mitochondrial division. *Science* **334**, 358–362 (2011).
- Görlitz, F. et al. easySLM-STED: stimulated emission depletion microscopy with aberration correction, extended field of view and multiple beam scanning. *J. Biophotonics* **11**, 1–11 (2018).
- Guichard, P., Chrétien, D., Marco, S. & Tassin, A. M. Procentriole assembly revealed by cryo-electron tomography. *EMBO J.* **29**, 1565–1572 (2010).
- Banterle, N. & Gönczy, P. Centriole biogenesis: from identifying the characters to understanding the plot. *Annu. Rev. Cell Dev. Biol.* **33**, 23–49 (2017).
- Janke, C. & Bulinski, J. C. Post-translational regulation of the microtubule cytoskeleton: mechanisms and functions. *Nat. Rev. Mol. Cell Biol.* **12**, 773–786 (2011).
- Bobinnec, Y. et al. Centriole disassembly in vivo and its effect on centrosome structure and function in vertebrate cells. *J. Cell Biol.* **143**, 1575–1589 (1998).
- Xu, Z. et al. Microtubules acquire resistance from mechanical breakage through intraluminal acetylation. *Science* **356**, 328–332 (2017).
- Portran, D., Schaedel, L., Xu, Z., Théry, M. & Nachury, M. V. Tubulin acetylation protects long-lived microtubules against mechanical ageing. *Nat. Cell Biol.* **19**, 391–398 (2017).
- Abal, M., Keryer, G. & Bornens, M. Centrioles resist forces applied on centrosomes during G2/M transition. *Biol. Cell* **97**, 425–434 (2005).
- Wong, Y. L. et al. Reversible centriole depletion with an inhibitor of Polo-like kinase 4. *Science* **348**, 1155–1160 (2015).
- Hamel, V. et al. Identification of chlamydomonas central core centriolar proteins reveals a role for human WDR90 in ciliogenesis. *Curr. Biol.* **27**, 2486–2498.e6 (2017).

39. Fortun, D. et al. Reconstruction from multiple particles for 3D isotropic resolution in fluorescence microscopy. *IEEE Trans. Med. Imaging* **37**, 1235–1246 (2018).
40. Sieben, C., Banterle, N., Douglass, K. M., Gönczy, P. & Manley, S. Multicolor single-particle reconstruction of protein complexes. *Nat. Methods* **15**, 777–780 (2018).
41. Jiang, J., Pentelute, B. L., Collier, R. J. & Hong Zhou, Z. Atomic structure of anthrax protective antigen pore elucidates toxin translocation. *Nature* **521**, 545–549 (2015).
42. Meehl, J. B., Bayless, B. A., Giddings, T. H., Pearson, C. G. & Winey, M. Tetrahymena Poc1 ensures proper intertriplet microtubule linkages to maintain basal body integrity. *Mol. Biol. Cell* **27**, 2394–2403 (2016).
43. Le Guennec, M. et al. A helical inner scaffold provides a structural basis for centriole cohesion. *Sci. Adv.* **6**, eaaz4137 (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 single 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 $\sim 10^5$ 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 μ l of the Lipofectamine 2000 reagent in 100 μ l of Opti-MEM medium per six wells.

For synchronization of RPE-1 cells at the G1/S transition and the onset of centriole assembly, $\sim 125,000$ cells were seeded for 24 h in six-well plates (Merck, TPP, 92006) on 12 mm coverslips in DMEM. Thymidine (Merck, T1895, 1 mM) and Centrinone (Lucerna-Chem, MCE-HY-18682, 300 nM) were added for 18 h, before fixation of cells with -20°C methanol for 5 min.

For immunofluorescence experiments with Cos-7 cells, cells were washed in prewarmed PBS before being fixed in prewarmed fixation buffer (4% paraformaldehyde in PBS). Cells were then permeabilized in 0.25% Triton-X in PBS for 10 min. After washing in PBS for 5 min, cells were incubated in blocking buffer (1% BSA in PBS) for 60 min. The primary antibodies (Tom20-rabbit (1:50, FL-145 sc-11415 Santa Cruz Biotechnologies), Tim23-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 10 min. Secondary antibodies (Alexa Fluor 488 goat antimouse IgG (H+L) (1:150, A28175 ThermoFisher), Alexa Fluor 568 goat antirabbit IgG (H+L) (1:150, A11011 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^{8,26}. 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 (F48–486, AHF Analysentechnik) and controlled through an acousto-optic tunable filter (AOTFnc-400.650-TN, AA Optoelectronic). In the case of Gaussian excitation, the beam was expanded with a 10 $\times$ beam expander ($f_{1a}=40$ mm, $f_{2a}=400$ mm, Thorlabs). In the mfFIFI path, a focusing lens ($f_{FC}=50$ mm) was used to focus the light near a rotating diffuser ($2.5^\circ \pm 0.25^\circ$ FWHM at 650 nm, 24-00066, Süss MicroOptics) before collimation by a collimating lens ($f_{CL}=60$ mm, Thorlabs). The collimated beam was contracted by a factor of four by two lenses ($f_{1b}=120$ mm, $f_{2b}=30$ mm, Thorlabs) before illuminating two flat-fielding MLAs (300 μ m pitch, 10×10 mm, $f=4.78$ mm, square lenses, 18-00157, Süss MicroOptics). The flat-field was then focused by a Fourier lens ($f_{FL}=300$ mm, Thorlabs). Both paths then illuminated the excitation MLA (222- μ m pitch, 1-inch diameter, $f=6$ mm, square lenses APO-Q-P222-R2.74, Advanced Microoptic Systems). The excitation was relayed by scan lenses ($f_{SL}=190$ mm, 55-S190–60-VIS, Special Optics) to and from the scanning mirror (SPO9086 Rev B Coated X Mirror, Sierra Precision Optics) mounted on a galvanometer scanner (QS-12, N-2071, Nutfield Technology). The excitation was then imaged onto the sample using a tube lens ($f_{TL}=350$ mm, 49-289-INK, Edmund Optics) and an objective lens (APON60XOTIRE, Olympus), resulting in a final magnification of roughly $\times 116$. The sample was placed on a precision aligned microscopy platform including a micropositioning control (RM21-AZ-AXY-RMS-M, Mad City Labs) and a z piezo stage (Nano-Z200, Mad City Labs). The emission was relayed back through the excitation side of the scanning mirror and split using a dichroic mirror (F58-488S, Semrock). The emission was then pinholed with a pinhole array (chrome on 0.090-inch-thick quartz, 222 μ m pitch, 40 μ m diameter, Photosciences) and relayed by two relay lenses ($f_8=300$ mm, Thorlabs) before contracting each emission spot by a factor of two using another MLA (222- μ m pitch, 1-inch diameter, $f=0.93$ mm, square lenses APO-Q-P222-R0.425, Advanced Microoptic Systems). The emission was then relayed using scan lenses on the emission side of the scan mirror, toward a filter wheel (Lambda 10-B, Sutter Instruments, Science Products) with two notch filters (NF-03-488E-25 and NF-03-561E-25, Semrock) mounted to block the excitation wavelengths. The transmitted emission was then collected by a sCMOS camera (PrimeBSI, 01-PRIME-BSI-R-M-16-C, Photometrics). The size of a square camera pixel corresponds to 56 nm on the sample.

The microscope was controlled using a custom written MATLAB script that controlled an analog output card (PCI-6733, National Instruments) and breakout box (BNC-2110, National Instruments) for precise control and synchronization of the scan mirror, AOTF, camera and z stage. The xy stage and camera were controlled through MicroManager⁴⁴.

For live-cell imaging, imaging was performed at 37°C in prewarmed Leibovitz medium in a top stage incubator (H301-PRIOR-NZ100-H117, Okolab). Imaging of immunostained samples was performed in PBS. Expanded samples were mounted as previously described²⁴. Briefly, the expansion factor was determined before imaging by measuring the gel size with a caliper (precision ± 0.01 mm). The gel was then cut using a razor, before completely removing excess water with a laboratory wipe and placing the gel on a poly-D-lysine treated coverslip (25 mm round coverslips, Menzel Gläser no. 1.5) already placed inside the imaging chamber. After gently pressing on the gel to ensure attachment to the coverslip, a few drops of ddH₂O were added on top of the sample to avoid shrinkage during imaging.

Image acquisition was performed using a custom written MATLAB script, in combination with MicroManager⁴⁴.

iSIM deconvolution. Raw iSIM images were deconvolved using the Lucy–Richardson deconvolution algorithm implemented in MATLAB and provided by H. Shroff⁴. Each raw z stack was deconvolved for 40 iterations.

mfFIFI characterization platform. An optical characterization setup was built to measure the variation in pitch and spot size of the multifocal points. A laser beam with a wavelength of 640 nm (CUBE 640-100 C, Coherent) was expanded with a 6 $\times$ beam expander ($f=50$ mm, $f=300$ mm, Thorlabs) with a pinhole (P50D, Thorlabs) at the joint focal plane of the two lenses to spatially filter the beam. The beam is passed through a Köhler integrator, made of the following components: focusing lens ($f=80$ mm, Thorlabs), rotating diffuser ($2.5^\circ \pm 0.25^\circ$ FWHM at 650 nm, 24-00066, Süss MicroOptics), collimating lens ($f=40$ mm, Thorlabs), two flat-fielding MLAs (300- μ m pitch, 10×10 mm, $f=4.78$ mm, square lenses, 18-00157, Süss MicroOptics), Fourier lens ($f_{FL}=300$ mm, Thorlabs). An iris (ID25, Thorlabs) is used before the two MLAs to control the portion of the beam, which is allowed to propagate forward to reach and illuminate MLAs, by adjusting the opening diameter of the iris. An excitation MLA (300- μ m pitch, 10×10 mm, $f=8.72$ mm, square lenses, 18-00221, Süss MicroOptics) is used to produce the multifocal points. The size of the multifocal illumination is reduced by a factor of 0.6 $\times$ by a beam de-expander ($f=50$ 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,h, 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 MLAs. Images of the beam at different iris opening diameters were analyzed using a MATLAB script, which allowed us to fit each excitation point to a two-dimensional Gaussian and measure the multifocal points' mean spot FWHM as function of iris diameter. To study the variation in 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 MLAs. 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 (telecentricity) 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 sample²⁷, as previously described⁴⁰. Briefly, sodium fluorescein (NaFITC) powder was diluted in deionized water, followed by vortexing and sonication until the powder was completely dissolved. A 10- μ l 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 spots 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 Fiji⁴⁵.

In the case of excitation spots visualized in Fig. 1f,g and the quantification in Fig. 1e,h, 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 Tetraspek 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

imaging axis). The profile was measured using a 7-pixel-thick line in Fiji⁴⁵. Each peak was fitted to a Gaussian profile to localize its center, before calculating the 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 Segmentation⁴⁶ and tracked by matching the closest neighboring center of mass positions between individual frames, as previously described⁴⁷.

Multiple FOV stitching. For stitching of multiple images, raw images or *z* stacks were acquired with a 10% overlap for both Gaussian and mFIFI excitation. The acquired images were then combined using the Grid/Collection stitching tool in Fiji⁴⁵ 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 uniform sample, but with enough features that it could be reliably stitched together. We used a dye lake sample (sodium fluorescein, NaFITC)²⁷, 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 mFIFI and Gaussian illumination, repeating the stitching for different stage displacements between adjacent FOVs (resulting in different degrees of overlap). We then stitched together the different 3 × 3 grids for mFIFI 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 mFIFI simulation platform has been adapted based on previous work¹⁰. 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- (ref. ⁴⁸) and expanded using the U-ExM protocol²⁴. Briefly, isolated centrioles were spun on 12 mm poly-D-lysine-coated (ThermoFisher, A3890401) 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 solution of 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 μ l sodium acrylate (Sigma-Aldrich, 408220, 38% (wt/wt, diluted with nuclease-free water)), 12.5 μ l acrylamide (Sigma-Aldrich, A4058, 40% stock solution), 2.5 μ l *N,N'*-methylenebisacrylamide (Sigma-Aldrich, M1533, 2% stock solution) and 5 μ l 10× PBS, supplemented with 2.5 μ l TEMED and 2.5 μ l APS (from 10% stock solutions). Once polymerized, gels were moved into denaturation 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 a 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 by 0.1% Tween20 (PBS-T) while shaking, followed by incubation with secondary antibodies for 3 h at 37°C in the 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 (1:2,000) 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 antipolyglutamate chain (polyE), pAb (IN105, 1:250) and mouse monoclonal anti-acetylated tubulin (Merck, T7451, 1:500). Secondary antibodies were goat antirabbit Alexa488 (ThermoFisher Scientific, A11034, 1:500) and goat antimouse IgG Alexa 568 F(ab')₂ (ThermoFisher Scientific, A11019, 1:500). The following primary antibodies were used for imaging expanded purified *C. reinhardtii* centrioles: rabbit polyclonal antipolyglutamate chain (PolyE, IN105, 1:500, AG-25B-0030-C050, Adipogen), mouse monoclonal anti- α -tubulin (DM1 α , 1:500, T6199, Sigma-Aldrich), mouse monoclonal antipolyglutamyl modification (GT335, 1:200, AG-20B-0020, Adipogen), mouse monoclonal anti-acetyl- α -tubulin (Lys40, 1:50, 32–2700, Invitrogen, ThermoFisher). Secondary antibodies were goat antirabbit Alexa Fluor 488 IgG H+L (1:400, A11008), goat antimouse Alexa Fluor 488 IgG H+L (1:400, A11029), goat antirabbit Alexa Fluor 568 IgG H+L (1:400, A11036), goat antimouse Alexa Fluor 568 IgG H+L (1:400, A11004 all from 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. The binarized points of interest were then dilated to form the mask that was then applied back to the raw stack. The particles were then cut out from the original stack with their specific mask and a 60 × 60-pixel surrounding region. The segmentation was ~50% successful, mostly because centrioles are often found in pairs, and closely located pairs had to be rejected since they could not be segmented correctly.

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 pairs 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. ³⁹, and followed the procedure reported in ref. ²⁴ with a C9 symmetry constraint. The point spread function was experimentally measured by imaging 100 nm fluorescent beads (TetraSpeck Microspheres, 0.1 μ m, T7279 ThermoFisher). We made two modifications to the reference-free step of the reconstruction algorithm³⁹. 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/3613906#.Xib5j1NKhtY>).

Code availability

The wave optics simulation platform for optimizing mFIFI is provided as Supplementary Software 1. Updated versions can be found on GitHub (https://github.com/dmahecic/simmla_w_mfifi). The custom MATLAB source code for aligning 3D particles is available at <https://github.com/dfortun2/U-ExM37>.

References

- Edelstein, A. D. et al. Advanced methods of microscope control using μ Manager software. *J. Biol. Methods* **1**, 10 (2014).
- Schindelin, J. et al. Fiji: An open-source platform for biological-image analysis. *Nat. Methods* **9**, 676–682 (2012).

46. Arganda-Carreras, I. et al. Trainable Weka Segmentation: a machine learning tool for microscopy pixel classification. *Bioinformatics* **33**, 2424–2426 (2017).
47. Mahecic, D. et al. Membrane bending energy and tension govern mitochondrial division. Preprint at *bioRxiv* <https://doi.org/10.1101/255356> (2018).
48. Klena, N. et al. Isolation and fluorescence imaging for single-particle reconstruction of *Chlamydomonas* centrioles. *J. Vis. Exp.* **139**, 58109 (2018).

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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 *C. 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 mFIFI 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>.

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Extended Data Fig. 1 | mfFIFI 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: $L_3 = F_{FL}$. 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 values of L_2 . Dashed line marks the actual pitch of the excitation MLA. **e**) Schematic representation showing the mFIFI 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: 1 μm .

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. α -tubulin signal was measured from three different datasets (dual-labeling with acetylated tubulin, GT335 and PolyE), with their individual means marked individually (acetylated tubulin in yellow, α -tubulin in blue, GT335 in orange and PolyE in magenta). **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: 1 μ m.

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.

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For all statistical analyses, confirm that the following items are present in the 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
- ☐ ☒ A statement on 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.
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Give P values as exact values whenever suitable.
- ☒ ☐ For Bayesian analysis, information on the choice of priors and Markov chain Monte Carlo settings
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- ☒ ☐ Estimates of effect sizes (e.g. Cohen's d , Pearson's r), indicating how they were calculated

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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- 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

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

Field-specific reporting

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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 no group allocation component to our study
Blinding	There was not a group allocation component to the study.

Reporting for specific materials, systems and methods

We require information from authors about some types of materials, experimental systems and methods used in many studies. Here, indicate whether each material, system or method listed is relevant to your study. If you are not sure if a list item applies to your research, read the appropriate section before selecting a response.

Materials & experimental systems

n/a	Involved in the study
☐	☒ Antibodies
☐	☒ Eukaryotic cell lines
☒	☐ Palaeontology
☒	☐ Animals and other organisms
☒	☐ Human research participants
☒	☐ Clinical data

Methods

n/a	Involved in the study
☒	☐ ChIP-seq
☒	☐ Flow cytometry
☒	☐ MRI-based neuroimaging

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 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 IgG Alexa 568 F(ab')₂ (Thermo-Fisher Scientific, A11019, 1:500)). For expansion of purified Chlamydomonas reinhardtii centrioles, primary antibodies used were rabbit polyclonal anti-polyglutamate chain (PolyE, IN105, 1:500, AG-25B-0030-C050, Adipogen), mouse monoclonal anti- α -tubulin (DM1 α , 1:500, T6199, Sigma-Aldrich), mouse monoclonal antipolyglutamylation modification (GT335, 1:200, AG-20B-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://www.bdbiosciences.com/us/applications/research/apoptosis/purified-antibodies/>

purified-mouse-anti-tim23-32tim23/p/611222
 -Monoclonal anti-acetylated tubulin 6-11B-1 (T7451) (used here in expanded human cells):<https://www.sigmaaldrich.com/catalog/product/sigma/t7451?lang=en®ion=US>
 -Anti-alpha-tubulin mouse monoclonal DM1A(T6199): <https://www.sigmaaldrich.com/catalog/product/sigma/t6199?lang=en®ion=US>
 - anti-Polyglutamylation Modification mAb GT335 (AG-20B-0020-C100) :<https://adipogen.com/ag-20b-0020-anti-polyglutamylation-modification-mab-gt335.html/>
 -PolyE antibody (IN105) recognizes specifically glutamate chains of four or more glutamates. <https://adipogen.com/ag-25b-0030-anti-polyglutamate-chain-polye-pab-in105.html/>
 -Acetyl-alpha Tubulin (Lys40) recombinant rabbit monoclonal antibody (RM318) (used here in purified Chlamydomonas reinhardtii centrioles): <https://www.thermofisher.com/antibody/product/Acetyl-alpha-Tubulin-Lys40-Antibody-clone-RM318-Recombinant-Monoclonal/MA5-33079>

Eukaryotic cell lines

Policy information about [cell lines](#)

Cell line source(s)	<p>RPE-1 cell line: purchased by PG from ATCC.</p> <p>COS-7 cell line: purchased by SM from HPA Culture Collections.</p>
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.