Volumetric interferometric lattice light-sheet imaging

Bin Cao¹², Simao Coelho¹², Jieru Li¹, Guanshi Wang¹ and Alexandros Pertsinidis¹*¹

Live cell imaging with high spatiotemporal resolution and high detection sensitivity facilitates the study of the dynamics of cellular structure and function. However, extracting high-resolution 4D (3D space plus time) information from live cells remains challenging, because current methods are slow, require high peak excitation intensities or suffer from high out-of-focus background. Here we present 3D interferometric lattice light-sheet (3D-iLLS) imaging, a technique that requires low excitation light levels and provides high background suppression and substantially improved volumetric resolution by combining 4Pi interferometry with selective plane illumination. We demonstrate that 3D-iLLS has an axial resolution and single-particle localization precision of 100 nm (FWHM) and <10 nm (1σ), respectively. We illustrate the performance of 3D-iLLS in a range of systems: single messenger RNA molecules, nanoscale assemblies of transcription regulators in the nucleus, the microtubule cytoskeleton and mitochondria organelles. The enhanced 4D resolution and increased signal-to-noise ratio of 3D-iLLS will facilitate the analysis of biological processes at the sub-cellular level.

Selective plane-illumination (light-sheet) approaches achieve the highest 3D spatial resolution and single-particle localization precision to date (<100 nm and <10 nm, respectively). Point-scanning 4Pi imaging, with focused excitation and confocal detection, features the most efficient 3D point spread function (PSF) in terms of reduced side lobes as well as background reduction. However, point scanning is limited in temporal resolution when imaging large fields, and short pixel dwell times require high peak excitation intensities that often accelerate photo-bleaching. Wide-field interferometric setups could, in principle, achieve faster imaging at reduced peak intensities. However, such setups typically have been implemented in an epi-illumination configuration, which suffers from increased out-of-focus background. Applications have, thus, been limited to relatively sparse and bright cellular structures with low background. There are many systems where structures of interest might comprise only ~10 molecules. Such structures are difficult to visualize in the presence of often overwhelming cellular background, such as out-of-focus blur in dense structures, as well as super-Poisson intensity noise due to particle number fluctuations. An approach that can simultaneously achieve high 3D resolution and localization precision and reduced background/photo-bleaching would greatly advance our capabilities to visualize molecular structures and motions at nanometer scales, in the high-background and crowded intracellular milieu.

Selective plane-illumination (light-sheet) approaches illuminate only a thin slice through the sample, overcoming many of the limitations of epi-illumination. In the simplest implementations, a single plane is illuminated with an excitation beam that is perpendicular to the detection optics. A conventional choice for creating the selective plane-illumination profile is a Gaussian beam, which can be projected by a separate excitation objective lens mounted perpendicular to the detection lens or reflected by a microfabricated cantilever mirror mounted on an excitation objective lens opposed to the detection lens. In either case, a tradeoff between the thickness of the light-sheet and the effective field of view due to diffraction needs to be considered, and a sweet spot is chosen depending on the requirements of the sample studied. To overcome the constraints due to diffraction of Gaussian beams, non-diffracting beams, such as Bessel or Airy beams, can be used. Both a single beam and an array of Bessel beams can be scanned to create a light-sheet that is thinner than what achieved with a Gaussian beam. However, the non-negligible excitation side lobes away from the main illumination plane introduce excess background and unnecessary photo-bleaching at out-of-focus parts of the sample that are not imaged. Selective plane-illumination based on bound 2D optical lattices, lattice light-sheet (LLS) illumination, can suppress the side lobes while maintaining the non-diffracting property and the thin profile of the light-sheet. LLS microscopy demonstrated combined low photo-toxicity, low photo-bleaching and low background, which are well-suited to live cell imaging studies. In conventional LLS imaging with a single 0.7 NA excitation lens and a single 1.1 NA detection lens, a 240 nm × 240 nm × 380 nm xyz resolution has been achieved, whereas, for single-particle localization applications, typical localization precisions are ~20 nm in xy and ~45 nm in z. This z performance is substantially lower than what can be achieved by interferometric methods, but, unfortunately, possibly due to the constraints of the dual opposed objective lens geometry, it has been challenging to implement selective plane illumination approaches in interferometric setups, to further reduce background and increase the achievable 3D resolution.

Here we report an interferometric imaging method for highly sensitive live cell imaging that replaces the original epi-illumination scheme with a selective plane-illumination scheme based on optical lattices (LLS illumination). This 3D interferometric lattice light-sheet (3D-iLLS) imaging method achieves a more confined detection volume than conventional LLS microscopy with a single detection objective lens and, thus, less out-of-focus background and higher signal-to-noise ratio (SNR). The reduced background, higher photon utilization efficiency and higher optical sectioning capabilities of 3D-iLLS enable visualizing weak sub-cellular structures. We demonstrate an achievable z resolution (100 nm (FWHM)) and...
localization precision (<10 nm (1σ)), both a factor of ~4x improvement compared to conventional LLS.

Results

Theory and experimental implementation of 3D-iLLS. To better understand and optimize 3D-iLLS microscopy, we developed a numerical simulation pipeline (Supplementary Note and Extended Data Fig. 1) for calculating the resulting 3D PSFs based on electromagnetic vector field calculations. The overall 3D-iLLS PSFs show distinct profiles compared to conventional LLS microscopy (Extended Data Fig. 2). In 3D-iLLS with constructive emission interference, the PSF exhibits a maximum centered at the common focus of the two objectives, with two additional visible side lobes along the z axis. When emission interferes destructively, the intensity maxima are symmetrically positioned along the z axis away from the focal plane, with two less pronounced side lobes. In both cases, the volume occupied by the overall PSFs for 4Pi detection is ≈2x less than for 2Pi detection. As the overall PSF becomes smaller, background contributions from molecules that diffuse in and out of the detection volume, or from molecules that bind to loci nearby, are reduced. This effect allows individual molecules and faint structures of interest at the focal plane to be visualized not only with higher z resolution but also with increased sensitivity and higher SNR.

Our 3D-iLLS microscope (Fig. 1a and Extended Data Fig. 3) uses two 1.1 NA opposed detection lenses, in a previously described interferometric cavity arrangement. For imaging, the interferometer is tuned near the zero-path-length position, resulting in
constructive/destructive interference at the two ports of the beam splitter. A third 0.7 NA lens, orthogonal to the two opposed detection lenses, delivers the LLS excitation. We calibrate the experimental 3D-iLLS PSF using 40-nm fluorescent beads (Fig. 1b). The experiment calibrations recapitulate our numerical calculations, featuring the expected modulated PSF with a ~100–140-nm FWHM central lobe, thus demonstrating successful implementation of the desired 3D-iLLS optical properties.

**Increased axial resolution of 3D-iLLS versus conventional LLS.**

To test the performance of 3D-iLLS with cellular samples, we visualized single mRNAs in human osteosarcoma cells (U-2 OS)13. Single mRNAs are tagged with PP7 phage-derived stem loops (24×PP7), visualized with a tandem-dimer phage coat protein fused to Halo-tag (tdPCP-Halo) and stained with a JF-646 Halo-tag ligand. 3D-iLLS adequately resolved single mRNAs, even in tight clusters where conventional LLS failed (Fig. 2a). We further quantified the axial resolution by measuring the z profile of single mRNAs. For conventional LLS, the obtained FWHM resolution is 496 ± 27 nm and 444 ± 80 nm for raw data and after de-convolution, respectively (mean ± s.d., n = 4). The 3D-iLLS z profiles after de-convolution yield a FWHM of 96 ± 10 nm (mean ± s.d., n = 4) (Fig. 2b). These results demonstrate a ≈4× axial resolution improvement of 3D-iLLS compared to conventional LLS.

To demonstrate the performance of 3D-iLLS in live cell imaging, we imaged mouse embryonic stem cells (mESCs) that are engineered with a SNAP-tag knocked into the endogenous Brd4 locus13. We previously showed that Brd4 forms foci containing ~15 tagged molecules at the enhancers of the Pou5f1 and Nanog genes13. Our 3D-iLLS imaging shows multiple Brd4 clusters throughout the...
nucleus of mESCs (Fig. 2c), suggesting extensive Brd4 clustering at mESC enhancers. 3D-ILLS resolves Brd4 clusters with increased resolution compared to conventional LLS (Fig. 2d). 3D-ILLS can also localize the center-of-mass of Brd4 clusters in the reconstructed cellular volumes with approximately 10-nm z localization precision (Extended Data Fig. 5). These results highlight the capabilities of 3D-ILLS for live cell imaging.

3D-ILLS-SIM provides improved optical transfer function contiguity and strength. An important consideration for

\[ \approx 10^{-nm} \]
Extended-resolution and super-resolution imaging techniques is faithfully reconstructing structures of all sizes, down to the resolution limit. Different spatial frequencies present within the support of the microscope’s optical transfer function (OTF) must be recovered above the noise level. OTF contiguity and strength are crucial, as it becomes challenging to recover signals in weak OTF regions. Interferometric 4Pi techniques, as well as selective plane-illumination microscopy techniques with modulated excitation (LLS, Bessel), often inherently contain OTF depressions, manifesting as side lobes in the effective PSF. Depending on the detailed OTF structure and the spatial frequencies present in the sample, de-convolution can minimize (for example, Extended Data Fig. 5a) or, in certain cases, even eliminate, side lobes. The dithered LLS parameters can be further optimized (Extended Data Fig. 5b–i), but we reasoned that, beyond merely assessing the appearance of the effective PSF (for example, based on side lobe strength), further evaluating the full OTF structure and seeking to increase OTF strength and contiguity, throughout its support in 3D Fourier space, would be beneficial for general 3D-ILLS imaging applications.

To achieve a more uniform and further extended 3D-ILLS OTF support, we explored 3D structured illumination microscopy (SIM). By virtue of shifting and adding multiple information components in Fourier space, SIM can potentially better cover different spatial frequency ranges. Comparison among theoretical OTFs reveals that, indeed, the combination of 3D-ILLS and 3D SIM (3D-ILLS-SIM) results in OTFs that feature substantially shallower depressions than 3D-ILLS with dithered LLS excitation (Extended Data Figs. 6 and 7). The corresponding 3D-ILLS-SIM PSFs also feature less prominent side lobes along the z axis than dithered LLS 3D-ILLS (Extended Data Fig. 7). Finally, the support of 3D-ILLS-SIM OTFs extends further along the x axis, featuring an (anisotropically) increased resolution, similarly to conventional LLS-SIM. These desirable optical properties predicted theoretically prompted us to further pursue and implement extended-resolution 3D-ILLS-SIM imaging.
To evaluate the capabilities of 3D-iLLS-SIM, we first visualized the microtubule cytoskeleton in COS-7 cells (Fig. 3a,b and Extended Data Fig. 8a). Consistent with the theoretical predictions, 3D-iLLS-SIM visualizes individual microtubules with improved z resolution. Quantification of individual filaments reveals a FWHM of 137 ± 14 nm versus 321 ± 23 nm and 579 ± 66 nm, for 3D-iLLS-SIM versus LLS-SIM and dithered LLS, respectively (mean ± s.d., n = 10, n = 7 and n = 4 individual z profiles). Similarly, 3D-iLLS-SIM achieves x: FWHM of 214 ± 35 nm versus 383 ± 18 nm for dithered LLS (mean ± s.d., n = 5 and n = 5 individual x profiles, respectively).

We further performed two-color 3D-iLLS-SIM imaging of mitochondria and microtubules, simultaneously resolving the hollow structure of individual mitochondria and the spatial relationships between mitochondria and microtubules (Fig. 4a–f and Supplementary Video 1). The resolution in the shorter wavelength used for imaging mitochondria is 116 ± 25 nm and 185 ± 50 nm in z and x, respectively (FWHM; mean ± s.d., n = 8 and n = 8 individual z and x profiles, respectively). Inspection of the Fourier transforms of reconstructed data further illustrates the increased resolution and the improved recovery of axial spatial frequencies of 3D-iLLS-SIM compared to 3D-iLLS using dithered LLS excitation (Extended Data Fig. 8b,c). Finally, we tracked mitochondria dynamics in live COS-7 cells using 3D-iLLS-SIM (Fig. 5a–c and Supplementary Videos 2 and 3) at ~1 min per volume temporal resolution and extended xz spatial resolution (143 ± 27 nm z FWHM and 224 ± 43 nm x FWHM; mean ± s.d., n = 14 and n = 16 individual z and x profiles, respectively). These results demonstrate the capabilities of extended-resolution 3D-iLLS-SIM imaging for multi-color applications and for volumetric time lapse imaging of sub-cellular dynamics.

Nanometer localization by 3D-iLLS modulation interferometry. Certain applications, such as single-molecule localization-based imaging and single-particle tracking, require fast 3D coordinate determination, in a narrow range near the focal plane, with sub-diffraction precision. Such localization measurements can greatly benefit from the reduced background in selective plane-illumination schemes, but conventional LLS microscopy with a single detection objective and astigmatism-based axial detection could achieve only ~40–50-nm z localization precision.3,4,19 We reason that the ~10x more efficient photon utilization efficiency of interferometric versus astigmatism-based localization and the 2x higher SNR of 3D-iLLS versus conventional LLS should push the 3D nanometer localization precision to the sub-10-nm regime.

To harness 3D-iLLS for improved z localization precision, we implement modulation interferometry16, a method that previously achieved ~1–2-nm z localization precision. We extract the z position by dynamically modulating the length of one of the interferometer arms and measuring the phase of the ensuing intensity modulation (Fig. 6a and Extended Data Figs. 9 and 10a). Previous modulation interferometry relied on the coherence of two counter-propagating excitation beams. Here, we instead rely on the coherence of the emitted fluorescent photons. Importantly, in the excitation-only interference configuration, the fluorescence signals in the two ports of the interferometer beam splitter are in-phase, whereas, for emission-only interference, the two signals are out-of-phase relative to each other. This effect, thus, enables measuring two phases simultaneously, one on each detection camera (Extended Data Fig. 9), which also improves the temporal resolution of modulation interferometry by two-fold (Fig. 6b and Extended Data Fig. 10b). Using the combined phases from Cameras 0 and 1, with a six-phase or four-phase modulation cycle, our 3D-iLLS setup achieves ~2-nm...
and ≈8-nm z localization precision, respectively (Fig. 6c,d and Extended Data Fig. 10c,d). This ‘open-loop’ performance, without any active stabilization, indicates short-term mechanical stability of the 3D-iLLS design in the <10-nm regime. 3D-iLLS and modulation interferometry also enabled successfully tracking the 3D movement of single 24× PPT mRNAs tagged with tdPCP-Halo-JEF646 in the cytoplasm of live U-2 OS cells (Fig. 6e). These results illustrate how 3D-iLLS can also be exploited for dynamic 3D single-particle tracking in live cells.

Discussion

Our results establish 3D-iLLS as a versatile technique with improved volumetric imaging of crowded cellular samples. 3D-iLLS increases the axial (z) resolution to ≈100-nm FWHM, compared to ≈400-nm FWHM using conventional dithered LLS (after de-convolution, at 640/700-nm excitation/emission wavelengths, respectively). 3D-iLLS-SIM features ≈180 nm × 250 nm × 120 nm FWHM x,y,z resolution compared to ≈180 nm × 250 nm × 310 nm FWHM x,y,z resolution for conventional LLS-SIM (both at 560/580-nm excitation/emission wavelengths, respectively). The volume of the overall PSF ovoid (≈2πr_x r_y r_z, where r_x, r_y, and r_z are the PSF half-width-at-half-maximum along x, y, and z, respectively) for 3D-iLLS-SIM as implemented here (NA 1.1 for detection and NA 0.65 for excitation) is almost identical to conventional 3D-SIM with high NA oil immersion lenses (NA 1.4, ≈130 nm × 130 nm × 320 nm FWHM x,y,z resolution)15, with both techniques featuring ≈2.8 × 10^15 µm PSF volume at 560/580-nm excitation/emission wavelengths, respectively. However, the selective plane-illumination of 3D-iLLS-SIM offers reduced out-of-focus background and photo-bleaching compared to epi-illumination of conventional 3D-SIM, which facilitates imaging of weak signals. The volumetric resolution is only 17% worse (≈2.8 × 10^15 µm versus ≈2.4 × 10^15 µm) than what was achieved by 3D LLS with non-linear SIM using patterned activation (3D PA NL-SIM; 118 nm × 230 nm × 170 nm FWHM x,y,z resolution, for the Skylan-NS fluorophore and 405/488/520-nm photoactivation/excitation/emission wavelengths, respectively)15. 3D-iLLS-SIM, compared to 3D PA NL-SIM, does not require specialized fluorophores or sample exposure to violet light.

Further developments are possible. 3D-iLLS and modulation interferometry3 could enhance 3D single-molecule localization-based super-resolution imaging, with the reduced background being particularly useful when imaging densely labeled samples14. The properties of 3D-iLLS could further be leveraged for future implementations of parallelized methods that use excitation patterns featuring intensity zeros (for example, 3D MINFLUX15) and for enhancing precision and reducing background in other modulation-enhanced localization microscopy approaches22-25, particularly for single-particle tracking and localization imaging in live cells.

Our current 3D-iLLS implementation exhibits adequate short-term stability for many applications. For prolonged time lapse observations or for localization-based super-resolution imaging requiring hours-long acquisitions, active stabilization, as we previously implemented for a 4Pi modulation interferometry setup3, could be further employed to ensure registration between the two opposed lenses and correct for phase shifts of the interferometer. Adaptive optics26 to correct for system- and sample-induced aberrations could further optimize the interferometric PSF properties, reducing the axial extent of the detection and excitation PSFs as well as better matching the detection PSFs to increase coherence and maintaining these optical properties for 3D-iLLS imaging of optically thicker, multi-cellular systems.

3D-iLLS-SIM offers increased OTF contiguity compared to 3D-iLLS with dithered LLS, but some ringing is still present in the final reconstructions. Additional de-convolution after SIM reconstruction might further improve image quality27. However, multiple reasons might underlie this remaining ringing: imperfect experimental OTF calibrations or imprecise illumination parameter estimation used for SIM reconstruction, as well as sample-induced phase differences between the two detection arms and subtle distortions of the LLS excitation pattern due to system- and sample-induced aberrations. Beyond affecting global image quality, such effects could also result in an effective OTF that is spatially varying throughout the sample volume. System aberrations could be corrected or accounted for by more detailed OTF calibrations, but sample-induced effects might be more difficult to predict a priori. More elaborate data acquisition and image reconstruction schemes could then be explored to mitigate such effects: use of spatially varying SIM reconstruction parameters38-39 and/or a spatially varying OTF40-43, for example obtained by OTF calibrations at multiple points of the field of view and/or data acquisitions with different phase shifter settings.

Our present 3D-iLLS implementation could also be easily modified for multi-color, super-resolution imaging that relies on single-molecule discrimination between spectrally overlapping dyes15-16. To image two spectrally well-separated dyes, we performed two-color imaging sequentially, refocusing the objective lenses after completing a z-stack for the first color. Quasi-simultaneous (for example, by interlacing different colors at each z position), multi-color 3D-iLLS live cell imaging and single-particle tracking could be achieved using fast adaptive optics, such as deformable mirrors, in the detection path, to enable real-time corrections of chromatic aberrations. The recently discovered Field Synthesis Theorem40 could also be explored as an alternative to dithered LLS, to possibly generate excitation patterns for 3D-iLLS that better fill the OTE. Finally, our design can be further augmented by introducing additional excitation lenses41 and implementing full 3D SIM schemes41, for eventual live cell 3D-iLS-SIM at sub-100-nm isotropic 3D resolution.

Online content

Any methods, additional references, Nature Research reporting summaries, source data, extended data, supplementary infor-
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Methods
3D-iLLS microscope setup. The 3D-iLLS setup is built on an actively stabilized vibration isolation platform (TMC STACIS ix) inside a temperature-controlled room. The 3D-iLLS microscope consists of an LLS excitation path orthogonal to the optical axis of the opposed detection lenses in a 4Pi interferometric arrangement. We use a custom 26x 0.7 NA NA objective lens (Special Optics, 54-10-7) and two x25 1.1 NA detection objective lenses (Nikon, MRD77225).

Detection path. The first detection lens is mounted on a 3D flexure stage driven by differential micrometers (Thorlabs, MBT616D). The second detection lens is mounted on a 3D flexure stage configured with differential micrometers and additional closed-loop piezoelectric actuators (Thorlabs, MAX301). An additional 50-mm travel stage driven by a stepper motor (Thorlabs, LNR505S) is used for coarse positioning of the second detection lens. The interferometric detection path is similar to our previous modification interferometry setup, including a motorized platform for coarse path length scanning and a piezoelectric phase shifter for fine-tuning/fast modulation (Physik Instrumente, S-303.CD, with E-709. CHG controller). The fluorescence beams from the two detection lenses interfere at a non-polarizing beam splitter, and the light from the two exit ports is filtered using quad notch filters (Semrock, StopLine N305-405/488/561/635-2E) and emission filters (Chroma, ET525/50, ET595/50 and ET700/75; selectable using a filter wheel; Thorlabs, FW103H), before it is imaged onto two sCMOS cameras (Hamamatsu, C11440-2CU) with two f = 50 cm achromatic lenses. Final magnification is ×100 nm per pixel.

Excitation path. The excitation path is built based on the originally reported LLS design. An AOTF device (AA OPTO-ELECTRONIC, ATFinC-400.650-TN and MPDSPC device) facilitates on/off switching of continuous-wave laser beams (488 nm, 560 nm and 642 nm; MPB Communications, 2RU-VFL-P-500-488-B1R, 2RU-VFL-P-500-560-B1R and 2RU-VFL-P-500-642-B1R or 2RU-VFL-P-2000-642-B1R, respectively) modulated by a phase-only spatial light modulator (SLM; Forth Dimension Displays, QXGA-3DM), an achromatic quarter-wave plate and a polarizing beam splitter cube. The modulated beam is Fourier transformed with a lens, and the unmodulated light is blocked using a custom annular mask (Photo Sciences, custom design). The resulting 2D optical lattice is dithered using an x-axis galvanometer (Thorlabs, GVS001), placed at a plane conjugate to the back focal plane (BFP) of the excitation lens. Sample and BFP conjugate cameras (Thorlabs, DCC1545M, and Edmund, EO-0312M) are used for inspection of the 2D lattice pattern.

Instrument control and synchronization. Instrument control and synchronization are achieved with a custom LabVIEW (National Instruments, 2015 64-bit) application and a field-programmable gate array-based real-time hardware system (National Instruments, PCIe-7852R LX30).

Sample mounting and sample cell. To position electron microscopy (EM) grids in the space between the three objective lenses, we machined a pincher-grip sample holder out of a stainless steel rod. The sample holder is mounted on a rotation mount and pitch-adjustable kinematic adapter (Thorlabs, RSPO5 and TPA01), which are further mounted on a 3D nanopositioning stage (Physik Instrumente, MPB Communications, 2RU-VFL-P-500-488-B1R, 2RU-VFL-P-500-560-B1R and 2RU-VFL-P-500-642-B1R or 2RU-VFL-P-2000-642-B1R, respectively) modulated by a phase-only spatial light modulator (SLM; Forth Dimension Displays, QXGA-3DM), an achromatic quarter-wave plate and a polarizing beam splitter cube. The modulated beam is Fourier transformed with a lens, and the unmodulated light is blocked using a custom annular mask (Photo Sciences, custom design). The resulting 2D optical lattice is dithered using an x-axis galvanometer (Thorlabs, GVS001), placed at a plane conjugate to the back focal plane (BFP) of the excitation lens. Sample and BFP conjugate cameras (Thorlabs, DCC1545M, and Edmund, EO-0312M) are used for inspection of the 2D lattice pattern.

Cell culture and staining. All cell cultures were maintained at 37 °C, in a 5% vol/vol CO2 atmosphere, in a humidified incubator. BRD9 OMG cells were cultured with +21 media with 400 μg ml−1 of G418 (Sigma-Aldrich, G8168-10ML) on a 0.1% gelatin-coated dish at 37 °C in a humidified 5% CO2 incubator. +2i media contained M-DMEM (Thermo Fisher Scientific, 10313021), 15% FBS (Gemini Bio, Bio, 50-100), 0.1 mM 2-mercaptoethanol (Thermo Fisher Scientific, 21985023), 2 μg ml−1 of penicillin–streptomycin, 500 μg ml−1 of non-essential amino acids (Thermo Fisher Scientific, 11140076), 1,000 μM of leukemia inhibitory factor (Millipore, ESG1107), 3 μM CHIR99021 (Millipore, Milp0129), and 1 mM D-PD0325901 (Axon Medchem, 1408). Before imaging, cells were stained with −2i media and deposited on 400 μg ml−1 of 4G18. For staining, cells were labeled with 0.3 μM SiR-BG for 10 min at 37 °C and then rinsed three times with new media.

CMV clone 5 cells were maintained in McCoy’s 5A media without phenol red (GE Healthcare, SH30200.01), supplemented with 10% FBS (Gemini Bio, Bio, 50-100), 1x non-essential amino acid solution (Thermo Fisher Scientific, 11140050), 1 mM sodium pyruvate (Thermo Fisher Scientific, 11360070), 100 μM of penicillin–streptomycin (Thermo Fisher Scientific, 15140122) plus 1 μg ml−1 of α-amanitin (Sigma-Aldrich, A2232) and 1 μg ml−1 of puromycin (Sigma-Aldrich, P8833). CMV clone 5 cells were nucleofected with 0.2 μg of tdPCP-Halo and 0.5 μg of TetR-RFP plasmids (Amaxa kit VCA-1003, Lonza) and seeded with media containing α-amanitin and puromycin. One-to-three days after nucleofection, cells were stained with JF646-SNAP-tag ligand (JF646-BG) and used for imaging experiments. For staining, cells were incubated with media containing 1µM JF646-BG for 1 h, rinsed once with new media and replaced with new media containing drugs.

Cos-7 cells were maintained in EMEM (ATCC, 30-2003 media), supplemented with 10% FBS and 1% penicillin–streptomycin (Thermo Fisher Scientific, 11140076) and then plated in EMEM media. One-to-four days after transfection, cells were seeded onto collagen-coated EM grids. Before live cell imaging, cells were incubated with EMEM media containing 1 µM JF646-Halo for 1 h, rinsed once with new media and placed in fresh media.

Cell fixation. Cells were fixed with freshly prepared 4% vol/vol methanol-free formaldehyde (Thermo Fisher Scientific, 28906) in 1× PBS at room temperature for 10 min and then rinsed three times with 1× PBS. After fixation, samples were stored at 4°C.

Cell immunofluorescence. EM grids seeded with COS-7 cells were briefly rinsed with PBS and then fixed with 3% vol/vol formaldehyde and 0.1% vol/vol glutaraldehyde in PBS for 10 min at room temperature. The fixed cells were further rinsed with PBS and quenched with freshly made 0.1% wt/vol sodium borohydride in PBS for 7 min. Next, the cells were permeabilized with blocking buffer (3% BSA and 0.5% Triton X-100 in PBS) for 10 min and stained with primary antibodies (mouse anti-β-tubulin, Thermofisher Scientific, 11120010; rabbit anti-α-tubulin, Sigma, A21185, 1:1000 dilution; goat anti-rabbit IgG at 1:5000, Cell Signaling, 5126, 1:1000 dilution) in blocking buffer and then rinsed with PBS three times for 10 min each. Finally, the sample was post-fixed with 3% vol/vol formaldehyde and 0.1% vol/vol glutaraldehyde in PBS for 10 min at room temperature and then stored in PBS at 4°C until imaging.
Two-color SIM imaging was performed sequentially for each color. The two colors were aligned and registered in 3D using TetraSpeck fluorescent bead fiducials attached on the EM grid. Live cell time lapse 3D-iLLS-SIM data were corrected for photo-blinking using exponential fitting. The volumetric data were imported in ImageJ for visualization and maximum intensity projection calculations.

3D-iLLS particle tracking using modulation interferometry. Raw images were imported in MATLAB and the frames from each modulation cycle were grouped together in a single maximum projection image. The maximum projection images were imported in MATLAB for 2D particle tracking analysis\(^7\). The trajectories of selected particles were further refined by performing a 2D Gaussian fit in 11 × 11 pixel regions of interest (ROIs) to obtain more accurate xy coordinates. For obtaining the z coordinate, we first sum the intensity of the pixels in a 7 × 7 pixel ROI centered on the xy coordinate of the particle. To combine the intensities measured from Camera 0 and Camera 1, we used a maximally symmetric fundamental hexagonal lattice (requiring five steps per modulation cycle). This results in considerable high-frequency noise.

Generation of LLS excitation patterns. For 3D-iLLS with minimal overall PSF side lobes, the thinnest achievable LLS excitation is desired. Based on our numerical calculations (Extended Data Figs. 1, 2, 5b–i, 6 and 7), we used a fundamental rectangular lattice, with wave vector corresponding to 0.59 NA and annular mask corresponding to NA range of 0.52–0.65. The main excitation peak is ~500 nm FWHM, with additional weaker excitation peaks above and below. For 3D-iLLS with excitation confined to a single plane and for single-particle tracking with modulation interferometry, we used a fundamental square maximally symmetric lattice, with wave vector corresponding to 0.6 NA or 0.64 NA and annular mask corresponding to NA range of 0.59–0.65. The main excitation peak is ~1-μm FWHM. For 3D-iLLS-SIM, as a tradeoff between speed (dictated by number of modulation cycles) and OTF uniformity, we used a maximally symmetric fundamental hexagonal lattice (requiring five stepped images), with wave vector corresponding to 0.58 NA and annular mask corresponding to NA range of 0.52–0.65.

3D-iLLS and 3D-iLLS-SIM volume reconstruction. Raw 3D-iLLS and conventional LLS data were de-skewed and de-convolved in MATLAB using the measured PSF. De-convolution was performed using the images in Fig. 2a, but not to the images in Fig. 2c,d. De-convolution was performed using ten iterations of the Richardson–Lucy algorithm with no damping. For reconstruction, only the constructive interference channel (Camera 0) was used, although future work could focus on how to best combine the information from both constructive and destructive interference channels for optimal reconstruction.

3D-iLLS-SIM reconstruction was performed with the original 3D-SIM algorithm\(^8\) implemented in MATLAB, with an experimental OTF calibration for each wavelength using 100-nm fluorescent TetraSpeck beads. Before 3D-SIM reconstruction, background was subtracted using a rolling bull filter\(^9\), which also removed the out-of-focus signal that could create high-frequency reconstruction artifacts\(^9\). For the SIM reconstruction parameters, the original algorithm was slightly modified as follows: the modulation wave vector \(\mathbf{p}\) was determined directly by peak-fitting the discrete Fourier transform (DFT) of the raw data. The DFT was calculated on a fine grid (ten-fold up-sampling factor compared to the 1256 pixels × 1256 pixel image data), in a small region in Fourier space close to the expected peak position, to obtain the modulation wave vector at sub-pixel precision. We found that the exact value of the initial modulation phase parameter, \(\phi_0\), did not considerably affect the reconstruction results; thus, instead of estimating it computationally from the raw data, \(\phi_0\) was set manually based on the approximate experimental value (determined by the x galvo position and adjusted (if needed) after inspecting the reconstructed data).

The Wiener parameter, \(w\), was set empirically, by inspecting the reconstructed data and balancing a tradeoff between resolution and SNR: too large \(w\) results in blurred images and gaps in the Fourier transform of the reconstructed data, whereas too small \(w\) results in considerable high-frequency noise.

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Author contributions
A.P. conceived, designed and supervised the study. A.P. and B.C. built the experimental apparatus. B.C. developed the data acquisition software, wrote analysis code and validated the optical performance of the 3D-iLLS setup. S.C. implemented 3D-iLLS-SIM techniques and performed experiments. J.L. developed the protocols for preparation and imaging of cell samples. G.W. performed numerical calculations. A.P. performed experiments, analyzed and interpreted the data and wrote the manuscript.
Competing interests
Memorial Sloan Kettering Cancer Center has filed patent applications (WO2018106678A1, 62/430117 and 63/070125) relating to this work, with A.P. and G.W. listed as inventors.

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Correspondence and requests for materials should be addressed to Alexandros Pertsinidis.

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Extended Data Fig. 1 | Numerical calculation of 3D-iLLS PSFs. a, Simulation pipeline. b, Near uniform sampling of 214 orientations viewed from three angles.
Extended Data Fig. 2 | Numerical 3D-iLLS PSFs for different excitation lattices and comparison with conventional LLS. Overall PSFs are calculated for 2π detection and for 4π constructive and destructive detection. Simulation parameters are given in Supplementary Tables 1 and 2.
Extended Data Fig. 3 | Three-objective 3D-iLLS configuration, liquid sample cell, sample holder and sample mounting geometry. 

a. Photograph of 3D-iLLS setup, highlighting the three-objective configuration, the liquid sample cell and the top-immersion sample holder.
b. Photograph of the pincher-grip sample holder with mounted EM grid.
c. Sample mounting geometry.
Extended Data Fig. 4 | Localization of Brd4 clusters in reconstructed 3D-iLLS images. a–d, Axial profiles of individual Brd4 clusters. Thick lines show non-linear least-squares fits to equations of the form $B + \frac{A}{2} (1 \pm \cos (k(z - z_0) + \theta)) e^{-\frac{(z - z_0)^2}{\sigma_z^2}}$ for Cam0 and Cam1, respectively. Global fitting is performed, with shared $k$, $\theta$ and $\sigma_z$ parameters. We obtain two separate localization measurements of the parameter $z_0$ that indicates the center position of the cluster, estimated independently from Cam0 and Cam1. e, Oscillation wave-vector $k$ is $0.02375 \pm 0.00059$ nm$^{-1}$ (mean±SD), indicating a relative error $\sigma_k/k$ of $\approx 2.5\%$. f, The center position $z_0$ shows a systematic offset between the two cameras of $dz_0 = 25$ nm and an r.m.s localization error $\sigma_{dz0} = 10$ nm. These systematic and random errors relative to the oscillation period ($2\pi/k = 265$ nm) are $\approx 9\%$ and $\approx 4\%$ respectively.
Extended Data Fig. 5 | See next page for caption.
Extended Data Fig. 5 | Reduction of axial side-lobes by deconvolution and optimization of the 3D-iLLS PSF. a, Reduction of side-lobes in 3D-iLLS imaging using deconvolution. Top: raw z profiles of two individual mRNA molecules, from the data in Fig. 2a. Bottom: z profiles of the same mRNAs, after 10 iterations of the Richardson-Lucy deconvolution algorithm with an experimental PSF. b-i, Optimized 3D-iLLS PSF based on a fundamental rectangular 2D bound lattice. b, SLM pattern and c, corresponding intensity at rear pupil. d Annular mask. e, Intensity at rear pupil after annular mask. f, Resulting 2D bound lattice in real space and g, corresponding dithered lattice excitation pattern. h, Axial profile of conventional LLS PSFs. i, Axial profile of 3D-iLLS PSFs. Cyan: excitation; yellow: detection; gray: overall.
Extended Data Fig. 6 | Comparison of OTFs obtained by conventional LLS, 3D-ILLS, conventional LLS-SIM and 3D-ILLS-SIM. Simulation parameters are given in Supplementary Tables 1 and 2.
Extended Data Fig. 7 | Line profiles of OTFs and PSFs obtained by conventional LLS, 3D-iLLS, conventional LLS-SIM and 3D-iLLS-SIM. a, conventional LLS and 3D-iLLS based on dithered LLS excitation. b, conventional LLS-SIM and 3D-iLLS-SIM based on SIM LLS excitation. Line profiles along the $k_z$ axis ($k_x=0$, $k_y=0$) and $z$ axis ($x=0$, $y=0$) are shown for OTFs and PSFs, respectively. Simulation parameters are given in Supplementary Tables 1 and 2.
Extended Data Fig. 8 | Resolution and recovery of spatial frequencies of conventional LLS-SIM vs. 3D-iLLS-SIM. a, Conventional LLS-SIM vs. 3D-iLLS-SIM of microtubules. Average z profile obtained from n=9 and 7 individual microtubules from the 3D-iLLS-SIM and conventional LLS-SIM data in Fig. 3a. b,c, Fourier transforms of 3D-iLLS-SIM vs. 3D-iLLS images of microtubules and mitochondria. Fourier transforms S(k) correspond to the real-space data shown in Fig. 4a. Maps show log(|S(k)|) in the k_xk_y and k_xk_z planes. 3D-iLLS data are obtained from the 3D-iLLS-SIM data by 5-phase averaging. Two experiments were repeated independently with similar results.
Extended Data Fig. 9 | See next page for caption.
Extended Data Fig. 9 | Illustration of z tracking using 3D-iLLS modulation interferometry with a 4-step modulation cycle. Data corresponds to part of the trajectory of a single mRNA molecule (shown in the second row of Fig. 4e). Top trace shows the displacement of the phase shifter. Four steps are taken, each corresponding to 1/4th of the interferometric period. The black and magenta traces show the intensity of the Cam0 and Cam1 images in each frame. In each step, two phases are measured simultaneously, one on each camera. The images from the first half of each original 4-step modulation cycle - corresponding to $\varphi=0^\circ$ and $90^\circ$ measured on Cam0 and $\varphi=180^\circ$ and $270^\circ$ measured on Cam1 - are combined in a single modulation cycle. Similarly, the images from the second half of the original 4-step modulation cycle - corresponding to $\varphi=180^\circ$ and $270^\circ$ measured on Cam0 and $\varphi=0^\circ$ and $90^\circ$ measured on Cam1 - are combined in a separate second modulation cycle. The blue line shows this combined Cam0+Cam1 intensity trace. The z position is then extracted by the phase of the intensity modulation, resulting in two successive z position measurements, one each for the first and second part of the original 4-step modulation cycle.
Extended Data Fig. 10 | Axial localization performance with 3D-iLLS and 4-phase modulation interferometry. a, Signals from a 40 nm bead on Cameras 0 and 1, over 100 4-step modulation cycles. The piezoelectric phase shifter is stepped in 182.5 nm increments, corresponding to 0°, 90°, 180° and 270° relative phases. Each step lasts 25 msec, for a total of 100 msec per 4-step modulation cycle. Right panel: zoom-in of the dotted region in the left panel, illustrating the anti-correlated signal modulation of Cam0 vs. Cam1. b, Signals from Cam0 and Cam1 are combined into a single modulation cycle, doubling the temporal resolution to 50 msec. Right panel: zoom-in of the dotted region in the left panel. c, Superposition of all 200 modulation cycles by collapsing the x axis in the interval [0-2π), showing excellent stability and reproducibility of the setup. Solid line: fit to a sine wave. d, Extracted phase and z coordinate, showing σ ≈ 8 nm r.m.s. localization precision. Two experiments were repeated independently with similar results.
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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 LabVIEW 2015 64-bit was used for all the data acquisition in the paper

Data analysis MATLAB 2014b and 2020a and ImageJ 1.50b and 1.53e were used for image analysis. Origin 8.5 was used for non-linear least squares curve fitting and graph plotting.

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Life sciences study design

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

| Sample size               | No sample size calculations were performed. Sample size was sufficient for demonstrating the performance of 3D-LLS vs. conventional LLS. |
|---------------------------|-------------------------------------------------------------------------------------------------------------------------------|
| Data exclusions           | No data were excluded from the analyses.                                                                                       |
| Replication               | Experiments were repeated at least two times independently, with reproducible results. Number of independent experiments is stated in the figure legends. |
| Randomization             | N/A                                                                                                                            |
| Blinding                  | N/A                                                                                                                            |

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| ☒ Eukaryotic cell lines         | ☒ Flow cytometry |
| ☒ Palaeontology and archaeology | ☒ MRI-based neuroimaging |
| ☒ Animals and other organisms   |         |
| ☒ Human research participants   |         |
| ☒ Clinical data                 |         |
| ☒ Dual use research of concern |         |

Antibodies

Antibodies used: Mouse anti-α-tubulin, Thermo Fisher Scientific 32-2500; Rabbit anti-Tomm20, Sigma HPA011562; Goat Anti-Mouse-IgG-Atto647N, Sigma 50185; Donkey Anti-Rabbit IgG-CF568, Sigma SAB4600076

Validation: Anti-α-tubulin (Thermo Fisher Scientific 32-2500) has been validated by western blotting and immunofluorescence, as stated in the manufacturer’s product page. Anti-Tomm20 (Sigma HPA011562) has been validated by the Human Protein Atlas project (www.proteinatlas.org), using immunofluorescence and western blotting.

Eukaryotic cell lines

Policy information about cell lines

Cell line source(s): U-2 OS and COS-7 cells were obtained from ATCC; C57/B6J mouse embryonic stem cells were obtained from Millipore Sigma.

Authentication: U-2 OS and COS-7 cells were originally authenticated by ATCC; C57/B6J mESCs were authenticated by karyotyping.

Mycoplasma contamination: Cells were tested negative for mycoplasma.

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