Super-Resolution Video Microscopy of Live Cells by Structured Illumination

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Abstract

Structured-illumination microscopy can double the resolution of the wide-field fluorescence microscope, but has previously been too slow for dynamic live imaging. Here we demonstrate a high-speed SIM that is capable of 100 nm resolution at frame rates up to 11 Hz for several hundred time frames. We demonstrate the microscope by video imaging of tubulin and kinesin dynamics in living *Drosophila* S2 cells in the total internal reflection (TIRF) mode.

The fluorescence microscope is an essential tool in many fields of biology, but, in its classic form, is incapable of spatial resolution better than about 200 nm because of diffraction. Several recent methods can go well beyond this limit^{1–6}, within the constraints of each technique. Localization-based methods such as PALM⁴, STORM⁵ and FPALM⁶ can reach extreme resolution levels by precisely localizing individual photoswitchable fluorophores, but require very large numbers of raw images – hundreds to tens of thousands – and are

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AUTHOR CONTRIBUTIONS

P.K. designed and built the rapid control system and wrote the manuscript draft. B.B.C. acquired and processed the data. L.W., B.B.C., and P.K. built optical hardware. E.R.G. prepared the samples. M.G.L.G. made the conceptual design and wrote the simulation. B.B.C. and M.G.L.G. analyzed data. B.B.C., M.G.L.G., and E.R.G. edited the manuscript.

therefore limited in speed. A recent incarnation of PALM decreases the acquisition time for a single frame from hours to around 25–60 seconds, and has recorded movies of ~20 frames; the downside is that only a subset of molecules are located per frame, which limits the effective resolution to ~60 nm⁷. Stimulated emission depletion microscopy (STED) has obtained resolution below 30 nm by de-exciting the edges of the illuminated scan spot through stimulated emission¹. STED has achieved an impressive frame rate of 28 frames per second at 62 nm resolution, though with low photon counts and over a relatively small field of view of 2.5×1.8 μm⁸. Enlarging the field of view would directly decrease the frame rate, because STED is a point scanning method. There is still a need for a technique that can combine spatial super-resolution with multi-Hz frame rates over large fields of view.

In structured-illumination microscopy (SIM), resolution is improved by moving high-resolution information into the normal resolution passband through spatial frequency mixing with an illumination pattern². It can improve resolution by a factor of two in its linear form, and by a larger factor if nonlinearity can be exploited³. Linear SIM achieves a resolution of about 100 nm, not quite as high as the above methods, but has potential for much higher frame rates than PALM because it requires fewer raw data images, and for much larger fields of view than high-speed STED at a given frame rate because it acquires pixels in parallel by wide-field imaging rather than sequentially by point scanning.

In microscopy in general, the highest frame rates are possible when the region of interest is thin enough that a single plane per time point suffices, rather than a focal series. Indeed, to our knowledge all super-resolution time series published so far have been two-dimensional^{7,8}. Total internal reflection fluorescence (TIRF) microscopy provides an extremely thin emitting region, which can be treated as 2D for SIM purposes. SIM has already been used in TIRF^{9–13}, but not for time series imaging of live samples. Here we demonstrate live TIRF SIM at 100 nm resolution, with 3.7 to 11 Hz frame rates over fields of view of 32×32 to 8×8 μm.

Our implementation of 2D SIM uses 9 raw images, acquired with different illumination patterns, to construct one high-resolution output image: a periodic pattern of parallel lines is shifted through three phases for each of three orientation angles². Our original SIM produced the pattern with a transmission phase grating that was translated by a piezoelectric actuator and rotated by a mechanical stage². The mechanical movement of the grating was slow and limited the acquisition speed to several seconds per frame. We have now decreased the pattern-switching time by three orders of magnitude by using a ferroelectric liquid-crystal-on-silicon spatial light modulator (FLC SLM) (Displaytech) to produce the patterns (Supplementary Fig. 1 online). To switch patterns one simply writes new digital image data to the SLM (Supplementary Fig. 2), which takes only 0.6 ms; the response time of the ferroelectric liquid crystal is even faster and does not limit the switching speed. The SLM consists of 1024×768 pixels, enough to illuminate the full field of view of our camera.

The pattern can be thought of as formed by interference of two collimated beams, created by diffraction from the SLM. For maximum signal the interference contrast must be maximized, which requires the two beams to be linearly polarized parallel to the pattern lines; the polarization must thus be co-rotated with the pattern orientation. We implement

polarization rotation with two custom FLC switchable phase retarders (Displaytech), see Supplementary Fig. 3. Their switching time of $<100\mu\text{s}$ is concurrent with SLM pattern switching, and does not add to the acquisition time. The time required for one raw data image is thus dominated by the readout time of the camera, or by the exposure time if it is longer. Our microscope used a 512×512 pixel frame-transfer EMCCD camera (iXon DV887, Andor Technology, Ltd.) with a maximum full-frame rate of 35 Hz; the corresponding maximum SIM frame rate is 1/9 of that, or about 3.9 Hz. When the full field of view is not needed, the SIM frame rate can be increased by reading out a subfield (e.g., 14.7 Hz at 128×128 pixels), or more drastically by using a camera with fewer pixels. The SIM reconstructions have twice as many pixels in each dimension as the raw data. Our current full field of view is $32\times 32\ \mu\text{m}$, but could be increased to 43×43 microns for the same camera without undersampling.

As a first demonstration, we imaged EGFP- α -tubulin in living *Drosophila* S2 cells, at illumination intensities of approximately $5\text{--}10\ \text{W}/\text{cm}^2$. To bring more microtubules into the region illuminated by TIRF, we used an established protocol that gently flattens the cells by mechanical pressure against a pad of agarose gel (see Methods). SIM produces a striking resolution improvement over conventional TIRF, as can be seen both in real space (Fig. 1a–c, Supplementary Video 1 online) and in frequency space (Fig. 1d–e, Supplementary Video 2). Isolated microtubules are reconstructed with a full-width-at-half-maximum of 112 ± 12 nm, compared to 275 ± 21 nm in the conventional images (based on 158 measurements on 8 data sets). On test samples with 100 nm fluorescent microspheres we have measured an average FWHM of 104 nm (data not shown).

Time series with hundreds of time points can be produced with this method, see Supplementary Videos 1, 3, and 4 for examples with 120, 200, and 480 time points respectively. One of these data sets (Supplementary Video 3) depicts microtubule dynamics in a tripolar mitotic spindle (multiple centrosomes are common in S2 cells). In the 480-frame data set, microtubule segments that were present in all frames photobleached by approximately 50%; segments that polymerized during the experiment were correspondingly less affected by bleaching.

To evaluate live SIM as a tool for studying microtubule polymerization and depolymerization dynamics, we imaged the area near a centrosome of a mitotic S2 cell (Fig. 2 and Supplementary Video 5). A useful way to visualize these processes is with kymographs, using the random variation in GFP labeling density along each microtubule (speckling) to track microtubule position and thereby distinguish overall movement of the microtubule from growth or shrinkage at the end. Because of its higher resolution, SIM can visualize speckling with enhanced clarity (Fig. 2c, Supplementary Video 6) and permits increased labeling densities that allow more precise localization of the microtubule end. Even if the signal-to-noise ratio in each time frame is low, the time series nature of the data allows true labeling density variations to be distinguished from noise in that they persist over time and move with the microtubules (Supplementary Fig. 5). Sharp transitions can be observed between states of steady polymerization or depolymerization, paused states of constant length, and states of slower or less stable evolution (brackets in Fig. 2c). With conventional microscopy (bottom), the speckling is much less sharp, and harder to

distinguish from background features such as the coarse horizontal stripes seen here, which are caused by exclusion of free monomeric EGFP-tubulin by organelles. The rates of steady polymerization and depolymerization that we see in such kymographs, 87 ± 26 nm/s and 267 ± 56 nm/s respectively (each averaged over 22 measurements), are comparable to values in the literature¹⁴ for S2 cells (107 ± 55 nm/s and 233 ± 75 nm/s).

The ability of live SIM to resolve single microtubules within the spindle and follow their individual movements and polymerization activity makes possible a new range of experiments. For example, it has been suggested that microtubules can nucleate from other spindle microtubules through an augmin-mediated pathway¹⁵. Live SIM should allow this process to be visualized directly. As a second example, microtubules in mitotic (or meiotic) spindles can exhibit “poleward flux” toward the centrosome, an incompletely understood phenomenon that has typically been studied at a collective level, for example by spot photobleaching of entire kinetochore fibers that contain multiple microtubules¹⁶, or by microinjection of dye-labeled tubulin at such low densities that different speckles likely belong to different microtubules¹⁷. With the higher resolution of SIM, poleward movement of spindle microtubules can be visualized and quantified at the single-microtubule level (Supplementary Fig. 6), and correlated with observed polymerization or depolymerization events at the microtubule end.

As a challenging test case with very rapidly moving structures, we imaged kinesin-73-EGFP in S2 cells. Kinesin travels actively along microtubules at a typical speed¹⁸ of about 780 nm/s, corresponding to one 100-nm SIM resolution distance in about 130 ms; to avoid artifacts the frame time should be comparable or shorter. A time series with 144 ms SIM frame time (16 ms exposures), acquired with a 256×256 pixel field, produced a clear reconstruction in which individual kinesin-cargo complexes can be followed along microtubule tracks (Fig. 3, Supplementary Videos 7 and 8). This data set was acquired at a nominal illumination intensity of ~ 26 W/cm², and faded by about a factor of 3 over 120 time frames. Those kinesin spots that appear to move progressively along tracks do so at speeds of 0.4–0.9 μ m/s, in reasonable agreement with expectations for kinesin. Still smaller fields of view allow even higher rates; over a 128×128 pixel field (which becomes 256×256 pixels after reconstruction) we were able to image kinesin dynamics at a frame rate of 11.1 Hz (Supplementary Video 9). The signal-to-noise ratio decreases at high speed, but the resolution is not severely affected: the average FWHM apparent size of persistent kinesin complexes in the 11-Hz data set was 112 ± 13 nm ($N=30$), identical to the average FWHM observed in the slower tubulin data.

A critical requirement in live SIM is that the image sequence for a given time point should be acquired in a time short enough that no fine sample features move by more than about one resolution length, to avoid reconstruction artifacts (Supplementary Fig. 7). For this reason, high acquisition speed is beneficial even in situations when observations are to be sparsely spaced in time (which may be desired in order to study a long-term process without observing so many times as to cause excessive photobleaching): each SIM sequence can be acquired rapidly enough to prevent artifacts, and successive sequences can be spaced out by appropriate delay times.

Our approach to live SIM could be extended in several ways. For example, multiple emission colors could be imaged simultaneously by adding cameras or split-view devices. The current design is limited to a single excitation wavelength (because the beam spacing, which must match the pupil diameter, is produced by diffraction and therefore proportional to the wavelength), but one excitation wavelength can excite multiple fluorophores with different emission bands¹⁹. Drastically higher frame rates would be possible by using faster cameras, though typically at a cost of either field of view or sensitivity. For example, existing 128×128 pixel EMCCD cameras could allow TIRF-SIM at over 50 frames per second over a $\sim 13 \times 13 \mu\text{m}$ area. It may be possible to reach even higher spatial resolution through nonlinear SIM³, but this requires a larger number of raw images per time frame and the use of photoswitchable dyes or fluorescence saturation methods, and puts greater demands on photostability. The hardware used here for 2D TIRF-SIM could also be used for 3D SIM²⁰ with only minor modifications: slightly different SLM patterns, and a different demagnification factor from the SLM to the sample (see Supplementary Note online). Live 3D SIM would have a slower frame rate than in 2D due to the larger number of raw images per time frame – at each time point it would use a focal series of axial planes spaced about 150 nm apart, and 15 instead of 9 raw images per axial plane²⁰ – and would therefore become less tolerant of sample motion, in proportion to the sample thickness. It may be quite promising on a class of relatively thin samples.

While linear SIM does not produce quite as high resolution as STED or PALM, its frame rate and number of time points exceed those of live PALM by an order of magnitude, and the area rates (product of frame rate and field of view) exceed those published for live STED by a similar factor. Speckle microscopy in the sense of Fig. 2c is not compatible with PALM as published, which images disjoint subsets of molecules, with statistically independent random variations, at different time points.

In summary, SLM-based SIM offers a combination of increased resolution, multi-Hz live imaging, long time series, and large field of view that other super-resolution techniques do not provide, and does so without requiring special fluorophores or extreme light intensities.

Methods

Cell maintenance and preparation

Drosophila S2 cells stably expressing EGFP- α -tubulin under the control of the Ac5 promoter were maintained as described²¹. Full-length KHC-73 was cloned from pooled cDNA, ligated into pENTR-D-Topo (Invitrogen) and inserted into a Gateway vector under the control of the metallothionein promoter²². S2 cells stably transfected with this construct were selected, and protein expression was induced with $20 \mu\text{M}$ CuSO₄ 24 hours before imaging.

To prepare cells for imaging, exponentially growing cells were resuspended in their conditioned media to a concentration of 2×10^7 cells/ml, and $30 \mu\text{l}$ of this suspension was pipetted onto a cleaned $24 \times 50 \text{ mm}$ #1.5 coverslip and covered with a piece of $170 \mu\text{m}$ thick 2% agarose gel, as described²³ except omitting the spacers. The agarose pad was covered

with a 22×22 mm coverslip, and sealed with Valap. Samples were imaged through the 24×50 mm coverslip.

Structured illumination

Excitation light (488 nm, from an argon laser (Innova 90C, Coherent)) was coupled via an acousto-optic deflector (AOM-40 AF, Intra-action) into a polarization-maintaining single-mode fiber (Oz Optics). The AO deflector was used as a fast shutter and intensity control. Light exiting the fiber was collimated and sent through a pattern generator (Supplementary Fig. 2) consisting of a 1024×768 pixel ferroelectric-liquid-crystal-on-silicon spatial light modulator (Displaytech), a polarizing beam splitter cube, and a half-wave plate. The light exiting the pattern generator was directed toward the microscope through a polarization rotator (Supplementary Fig. 3) consisting of two custom ferro-electric liquid crystal phase retarders with $1/3$ -wave retardance (Displaytech) and a quarter wave plate. Unwanted diffraction orders, caused by the finite-sized pixels of the SLM (Supplementary Fig. 4), were blocked by a mask located in a pupil plane. The desired ± 1 diffraction orders were refocused to points near opposite edges of the back focal plane of the microscope objective. After being recollimated by the objective lens ($100\times/1.49$ TIRF, Nikon), each beam approached the cover slip surface at an angle larger than the critical angle for total internal reflection. Evanescent waves from the two beams interfered to produce a line pattern of excitation intensity. Fluorescent emission light from the specimen was directed toward a camera by a dichroic mirror, as in a conventional fluorescence microscope. The dichroic mirror (Chroma) used a custom coating with minimal transmission retardance at the excitation wavelength, to maintain the desired polarization state; was deposited on an 3.2 mm thick optically flat substrate to minimize aberrations in the emission light; and was operated at 22.5° to minimize aberrations in the excitation light.

The spatial light modulator (SLM) was operated as a binary phase modulator. Each pixel producing a phase shift of nominally zero or π radians, if written to with a binary 0 or 1 respectively.

To optimize the beam spacing in the pupil plane to match the TIRF zone diameter (see Supplementary Fig. 4), the SLM projection lens (the lens located between the beam splitter cube and the polarization rotator in Supplementary Fig. 1) was implemented as a lens pair consisting of an $f = 350$ mm achromat and an $f=7500$ mm singlet; the effective focal length of the combination could then be adjusted by changing the distance between the two lenses. The SLM pixel pitch, referred to sample space, is about 60 nm; the illumination pattern line spacing corresponds to 3 SLM pixels, or approximately $0.18 \mu\text{m}$. The 1024×768 pixels of the SLM thus suffice for illuminating a field of $61 \times 46 \mu\text{m}$, which is larger than the field of view of the camera: 512×512 pixels of size 63 nm, covering a field of $32 \times 32 \mu\text{m}$.

The ferroelectric liquid crystals are driven with a DC electric field – with opposite directions for the “on” and “off” pixel states – which must time-average to zero in order to avoid slow deterioration of the device through charge migration. Each pixel must thus spend as much time “on” as “off.” To this end, each pattern was polarity-switched (switching “on” pixels to “off” and vice versa) halfway through each raw exposure; both polarities produce the same

illumination pattern in the sample. The illumination light was turned off by the acousto-optical shutter while a new pattern was being written to the SLM.

The illumination intensities quoted in the main text are the nominal ones: incident power divided by the field of view. The actual photon flux in the TIRF zone (given by the Poynting vector, which in the TIRF zone is parallel to the interface) varies rapidly with depth, reaching ~4–6 times the nominal intensity immediately adjacent to the cover glass, and decreasing exponentially with depth with a decay constant of ~100 nm.

System Control

The desired patterns for one time point were preloaded onto the SLM controller PCI card. A digital signal processing card (M67, Innovative Integration), running a program based on hardware-timed interrupts, output timed TTL signals to gate the acousto-optic shutter, trigger the camera to initiate frame transfer, switch polarity on the liquid crystal retarders in the polarization rotators, and trigger the SLM controller to load the next pattern onto the SLM.

Transfer function

An empirical point spread function (PSF) was measured by imaging a 100-nm-diameter in-focus fluorescent microsphere (Yellow-Green Fluospheres, Invitrogen) dried onto a cover glass and mounted in water. An optical transfer function (OTF) was calculated by 2D Fourier transforming the background-subtracted PSF, dividing out the phase gradient corresponding to the bead position, and rotationally averaging the result.

Acquisition

At each time point, raw SIM data were acquired at phases 0, $2\pi/3$, and $4\pi/3$ of the illumination pattern, for each of the three pattern orientations. The back-illuminated EMCCD camera (iXon DU-897 BI, Andor) was operated in frame transfer mode at -78°C with water cooling (to avoid fan vibrations), and read out at 10 MHz with 14 bit digitization. The digital parameter that controls the electron multiplication gain (and which for this camera is nonlinearly related to the actual gain factor) was adjusted for each data set depending on sample brightness, typically in the range 2000–3000.

Data processing

SIM data were reconstructed as described^{2,20}. The process, done independently on each time point, is summarized below.

Preprocessing—As a precaution against edge-related artifacts, the raw data were typically preprocessed by slightly intermixing opposing lateral edges (the outermost ~10 pixels).

Where not indicated otherwise, all raw data images in each time series were rescaled to equalize their total background-subtracted intensity, to compensate for light source intensity fluctuations and photobleaching. Because of its low signal and high level of background

noise, the fastest kinesin data set (Supplementary Video 8) was instead bleach-corrected based on curve-fitting to the whole sequence.

Separation—For each pattern orientation d , the three images taken at different illumination phases were each Fourier transformed spatially, and then “separated” (Fourier transformed with respect to the phase shift variable), resulting in three information components $D_{d,m}$ representing information that had been moved in frequency space, through mixing with the illumination pattern, by vectors $-m\mathbf{p}$, where $\mathbf{p}$ is the spatial frequency of the illumination pattern and $m = -1, 0$, and $+1$ for the three components.

Parameter fitting—For each pattern orientation d , precise values of the pattern wave vector $\mathbf{p}_d$, the starting phase, and the modulation depth were determined from the data by comparing the $m = 1$ and $m = 0$ components in the region of frequency space where they overlap. To equalize the scaling so as to allow direct comparison, each component was first multiplied by a version of the OTF that had been shifted to be centered at the position of the other component. The two components were then cross-correlated in three steps: first a standard fast-Fourier-transform-based cross-correlation in frequency space (yielding values only at discrete frequency-space pixels), then parabolic interpolation to locate the peak of that cross-correlation to sub-pixel accuracy, and finally refinement through an optimization search in which sub-pixel frequency-space shifts were applied in the form of real-space phase gradients. The location of the cross-correlation peak yields the shift vector $\mathbf{p}_d$; the modulation depth a_d and starting phase ϕ_d were then found by complex linear regression on corresponding pixel values from the $m = 1$ and $m = 0$ components²⁰.

In the time series reconstructions presented here, these parameters were independently fit to the data for each time point. In practice, we have not observed any significant drift of the pattern parameters within any data set. It would thus be possible to do global parameter fits on the entire data set. Doing so would make the fit more robust against noise, and could improve the reconstruction quality drastically when the signal-to-noise ratio of the raw data is low. Some parameters, such as the pattern angles and line spacing, may in fact be stable enough that they can be assumed known without any fitting.

Reconstruction—Once the parameters were determined, the different information components were combined through a generalized Wiener filter:

$$R(\mathbf{k}) = \frac{\sum_{d,m} O(\mathbf{k} + m\mathbf{p}_d) a_d e^{-i\phi_d} D_{d,m}(\mathbf{k} + m\mathbf{p}_d)}{\sum_{d',m'} |O(\mathbf{k} + m'\mathbf{p}_{d'}) a_{d'}|^2 + w^2} A(\mathbf{k}) \quad (1)$$

where $R(\mathbf{k})$ is the value of the reconstruction (i.e. the estimate of the true sample information $S(\mathbf{k})$) at the point $\mathbf{k}$ of frequency space, the sums are taken over the 3 pattern orientations and the 3 information components for each orientation, w^2 is the Wiener parameter (which was taken to be a constant and adjusted empirically), and $A(\mathbf{k})$ is an apodization function (typically a 2D triangle function, which decreases linearly from unity at the origin to zero at the extended resolution limit).

To avoid amplifying edge effects, Eq. 1 was implemented in the manner described in reference²⁰: each un-shifted information component was separately multiplied by a filter function representing the other factors in its term of Eq. 1; the filtered results were then padded with zeros (to provide space for shifting information by the pattern vectors $\mathbf{p}_d$, or equivalently to decrease the real-space pixel size to accommodate the increased resolution), transformed to real space, multiplied by the complex phase gradient $e^{2\pi i \mathbf{p}_d \cdot \mathbf{r}}$ (which represents the frequency-space shift by the pattern wave vector $\mathbf{p}_d$), and added together, to produce the final reconstruction

Image analysis and figure preparation

Image analysis was done with the Prism software package developed at the University of California, San Francisco. The final figures were assembled in Illustrator (Adobe).

Conventional images for comparison were synthesized from the SIM raw data by, at each time point, summing the raw data images for all orientations and phases. Doing this, as opposed to acquiring a separate conventional data set, assures that the comparisons between conventional and SIM images are done for exactly equivalent conditions of excitation exposure, photobleaching, etc.

The kymographs in Figs. 2c and 3e were produced by a horizontal maximum-intensity projection of the indicated areas for each time point. The 3D kymograph Fig. 3d was produced by treating the xyt data set as a 3D volume, and making maximum-intensity projections through this volume along a viewing direction in xyt space. The 3D kymograph video in Supplementary Video 5 was produced by repeating the same process multiple times while successively rotating the viewing direction vector around the time axis.

The Fourier space images in Figs. 1d,e and Supplementary Video 2 were displayed using a nonlinear gray scale (gamma ~0.4), and with the high values near the origin truncated, in order to visualize clearly the weaker high-resolution data.

In preparing Supplementary Videos 1, 3, 4, and 5, the SIM reconstructions were binned 2×2 , thus reducing them from 1024×1024 to 512×512 pixels, and the data sets slightly cropped, to avoid excessively large file sizes. Because the raw image data were somewhat oversampled as acquired (a pixel size of 63 nm, compared to the Nyquist limit of 84 nm based on the objective's NA, and of about 120 nm based on its effective resolution), the pixelation of the binned videos is still sufficiently fine that the increased resolution can be appreciated. The data for Supplementary Video 7 were similarly binned from 512×512 to 256×256 pixels. For Supplementary Video 9, which covers a smaller area, the conventional images were instead up-sampled to match the full-resolution SIM reconstruction. Supplementary Video 2 was prepared by Fourier-transforming the full-resolution, full-field data, and binning the result 2×2 in frequency space. The 3D kymograph in Supplementary Video 6 was generated by first producing full-resolution projections through the full-resolution data volume, and then binning the resulting video 2×2 .

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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Figure 1.

Comparison of conventional TIRF (**a**) and TIRF-SIM (**b**) images of the microtubule cytoskeleton in a single S2 cell. Scale bar 2 μm . (**c**) Normalized intensity profiles along the yellow lines in (**a**) (red curve) and (**b**) (blue curve). Two microtubules separated by $\sim 150\text{ nm}$ are well resolved in the SIM reconstruction, but not by conventional microscopy. (**d–e**) Fourier transforms of the images in (**a**) and (**b**) respectively. The classical diffraction limit of the objective lens is indicated by a dashed circle of radius $5.96\text{ }\mu\text{m}^{-1}$. Sample information is visible as a bright “starburst” in the low-spatial-frequency central region of (**d**). That it does not reach the diffraction limit indicates that the effective resolution is lower than theory predicts, as is true for most high-NA objectives. The same sample information features can be recognized in (**e**), where they continue out to significantly higher spatial frequencies, well beyond the diffraction limit. Time series videos of (**a,b**) and (**d,e**) are available as Supplementary Videos 1 and 2 online.

Figure 2.

Time series live TIRF-SIM of EGFP- α -tubulin in an S2 cell. (a) Subset of one time frame (number 95) from a 180-frame sequence. Each frame was acquired in 270 ms (i.e., a raw data exposure time of 30 ms), using the full 512×512 pixel field of view of the camera. Time frames were deliberately spaced at 1 second intervals. (b) The green-boxed area of (a) shown at selected times as indicated, using conventional TIRF (left) or TIRF-SIM (right). The contrast of the conventional images has been increased for clarity. Green arrows indicate the end of one particular microtubule, which can be seen elongating until approximately the 100 s time point, and then rapidly shrinking; these changes are much easier to follow in the SIM reconstruction. (c) Maximum-intensity kymographs, using TIRF-SIM (top) and conventional TIRF (bottom), of the red-boxed area of (a). Five separate microtubules are seen expanding and contracting through the area. One microtubule in particular is retracting noticeably, causing tilted lines in the kymograph (blue arrow). Sharp transitions separate periods of steady polymerization or depolymerization from periods of constant length (examples indicated by yellow and orange brackets), or of slower and less stable growth or shrinkage (red bracket). Some constant-length periods are followed by resumed polymerization (orange bracket), others by rapid depolymerization (yellow bracket). Scale bars, 2 μ m. A time series video and a three-dimensional kymograph of this data set are available as Supplementary Videos 5 and 6 online.

Figure 3.

Time series live TIRF-SIM of kinesin-73-EGFP in an S2 cell. **(a–b)** Conventional TIRF **(a)** and TIRF-SIM **(b)** images of the first of 120 time frames. Each frame was acquired in 144 ms (i.e., a raw data exposure time of 16 ms), using a 256×256 pixel field of view. **(c)** Projection of the first 20 TIRF-SIM time frames, color-coded for time. Moving and stationary kinesin-cargo complexes can be seen as multi-colored curves and white spots respectively. **(d)** 3D maximum-intensity kymograph of the area red-boxed in **(b)**, covering the first 80 time frames. Kinesin spots exhibited a variety of behaviors, including clustering together (red arrowhead), splitting or separating (yellow arrowhead), remaining stationary (vertical line between the green arrowheads), and traveling at a constant velocity (inclined straight line between the blue arrowheads). **(e)** Kymograph produced by maximum-intensity-projecting the area blue-boxed in **(b)**, for the first 46 time points, onto the y-t plane. A traveling kinesin complex (red arrow) joined a nearly stationary kinesin complex (bottom center), and halted. After a 0.6 s pause, one kinesin complex traveled onward (yellow arrow), while the other remained stationary for another 1.8 s, at which time it also resumed forward travel (green arrow). A third kinesin complex that traveled past later, possibly along the same microtubule, did not pause (blue arrow). Scale bars, 2 μm **(a–c)** and 0.5 μm **(e)**. Videos of this data set are available as Supplementary Videos 7 and 8 online.