Whole-cell, 3D and multi-color STED imaging with exchangeable fluorophores

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Supplementary Information

Experimental Methods

Cell Culture

E. coli K12 MG1655 wildtype cells (CGSC# 6300) were streaked onto LB plates lacking antibiotics. Single colonies were picked and used to inoculate LB overnight cultures. Overnight cultures were then diluted 1:200 into fresh LB and incubated at 32°C and 230 rpm for aeration. For click-labeling of nascent DNA, EdU (Baseclick) was added to the culture (OD_{600} ~ 0.2 - 0.25) for 30 min to a final concentration of 10 µM.

Eukaryotic cells (HeLa Kyoto or HeLa ATCC CCL-2) were cultured at 37°C and 5% CO_2 in DMEM containing 4.5 g/l glucose, 10% FBS and 1% GlutaMAX (all purchased from Gibco). HeLa ATCC cells used for fixed-cell imaging were seeded on 8-well chamberslides (Sarstedt, 1-2 x 10^4 cells/well) which were previously coated with 15 µg/ml fibronectin (Sigma Aldrich) for 30 min. For live-cell experiments except (photo)toxicity experiments (see section live-cell imaging), HeLa Kyoto cells were seeded on Mattek chambers (#P35G-1.5-10-C) 24-48h before imaging.

Sample preparation

After reaching mid exponential phase (OD_{600} ~ 0.5), aliquots of *E. coli* cultures were fixed in solution for 12-15 min using a mixture 2% methanol-free formaldehyde (ThermoFisher) and 0.05% EM-grade glutaraldehyde (Electron Microscopy Sciences) in 33 mM sodium phosphate buffer pH 7.5. Cells were

pelleted by centrifugation (2 min, 6.000 g) and resuspended in PBS containing 0.2% sodium borohydrate to quench excess aldehydes. After 3 min quenching, cells were washed 3 times with PBS (centrifugation and resuspension for each step) and immobilized on KOH-cleaned (3M, 30 min) and poly-L-lysine (Sigma) coated 8-well chamberslides (Sarstedt). Cells were permeabilized using 0.5% Triton X-100 (Sigma) in PBS for 40 min and subsequently rinsed 2x with PBS.

Click-labeling of nascent *E. coli* DNA using JF_{646} azide or ATTO647N azide (ATTO-TEC) was performed as described elsewhere (Spahn, Endesfelder et al. 2014). For WGA-labeling, fixed and permeabilized cells were blocked for 30 min using Image-iT FX signal enhancer (ThermoFisher) and further 30 min using 2% BSA (Carl Roth) in PBS. 0.1 mg/ml WGA-AF594 (ThermoFisher) in PBS was added to the cells for 2h, followed by 5 washing steps using PBS and 15 min post-fixation using 2% FA in PBS. Excess FA was quenched by 5 min incubation with 50 mM ammonium chloride (Sigma) in PBS. Tetraspeck Fluorospheres (0.1 µm, ThermoFisher) were added to all *E. coli* samples.

HeLa cells used for STED imaging of membranes/DNA were fixed using 4% methanol-free FA in PBS for at least 75 min. Cells were washed once with PBS and excess FA was quenched using 50 mM ammonium chloride in PBS for 20 min. Cells were not permeabilized in order to preserve cellular membranes.

Hela cells used for imaging of the actin cytoskeleton were fixed using 0.5% glutaraldehyde (Electron Microscopy Sciences) for 10 min after extracting the cytosol thrice (1 min each) using a microtubule-stabilizing buffer containing 0.5% Triton-X 100. Excess glutaraldehyde was quenched using 0.2% sodium borohydrate (Sigma) in PBS for 6 min. For us, this protocol provided better results compared to extraction and fixation using a cytoskeleton buffer (Cramer and Desai: Fluorescence Procedures for the Actin and Tubulin Cytoskeleton in Fixed Cells).

STED Microscopy

STED images were acquired with two different commercial microscopes.

An inverted TCS SP8 3X microscope (Leica Microsystems, Mannheim Germany) equipped with a 100x/1.4 NA oil immersion objective (Leica HC PL APO CS2 - STED White) or a 86x/1.2 NA water immersion objective (Leica HC PL APO CS2 - STED White) was used for the majority of experiments, operated by the Leica LAS X software (version 3.1.5.16308). Fluorophores were excited with 561nm or 646nm laser light derived from a 80 MHz pulsed White Light Laser (Leica Microsystems, Mannheim Germany) and the stimulated emission was performed with a 775 nm pulsed laser (Leica Microsystems, Mannheim Germany). The fluorophore emission was collected with Hybrid Detectors (HyD, Leica Microsystems, Mannheim Germany) using a gate of 0.3-6 ns in respect to the excitation pulse. For single-color Nile Red imaging, a typical spectral window of 570-700 nm was used. For dual color Nile Red and JF_{646}-Hoechst imaging, two spectral windows of typically 570-

635 nm and 650-730 nm (up to 750 nm) were applied and images were recorded in line sequential mode using 561nm and 646nm excitation, respectively. Pixel size was set to 20 - 30 nm for 2D-STED microscopy and ~40 nm for 3D-STED microscopy if not stated otherwise. The pinhole was set to 0.6-1.0 AU for 2D-STED microscopy and closed down to minimum 0.54 AU in 3D-STED. Images were typically recorded in photon counting mode or standard mode using 2-16x line accumulation or line averaging, respectively. Dwell times were kept in the range of 0.2-2.43 µs. The microscope was equipped with an incubation chamber (constructed in-house at EMBL Heidelberg) and a temperature of 22.5 ± 0.2 °C was ensured by constant cooling to minimize sample drift and optimize optical performance. Exact parameters for each measurement are listed in Supplementary Table 1.

Alternatively, STED imaging was performed on a combined Abberior STED and RESOLFT system (Expert line; Abberior Instruments, Göttingen, Germany) operated by the Imspector software (v0.13.11885; Abberior Instruments, Göttingen, Germany). Samples were imaged with an Olympus UPLSAPO 60x NA 1.2 water immersion objective (UPLSAPO60XW/1.2; Olympus, Japan) on an Olympus IX83 stand (Olympus, Japan). The microscope was equipped with an incubation chamber (constructed in-house at EMBL Heidelberg) for temperature control. A temperature of 22.5 ± 0.2 °C was ensured by constant cooling to minimize sample drift and optimize optical performance. Nile Red was excited with a 594 nm pulsed laser and JF_{646}-Hoechst with a pulsed 640nm laser. Stimulated depletion was performed with a 775nm pulsed laser. Imaging was hereby performed in line sequential mode. The fluorescence signal was detected on Avalanche photo diodes (APDs) with bandpass filters of 605-625nm for Nile Red and 650-720nm for Hoechst JF-646. A gating of 0.78ns - 8 ns was applied. The pinhole was set to 2.0 airy units and the pixel dwell time to 10 µs. For the confocal image, each line was scanned once. For the STED image, each line was scanned 5 times and the signal was accumulated.

JF_{646}-Hoechst, SiR-Hoechst (Spirochrome) and Nile Red (Sigma, catalogue number N3013) stock solutions (100 µM in DMSO for Hoechst conjugates and methanol for Nile Red) were diluted in 150 mM tris pH 8.0 (ThermoFisher). For imaging, a final concentration of typically 300 nM was used. 1 mM stock solution of FM4-64 (ThermoFisher) in PBS was diluted in 150 mM tris pH 8.0 to a final concentration of 1 µM. Synthesized Alexa-Fluor 594 conjugated Lifeact (sequence: MGVADLIKKFESISKEEGSGS[C]; the C-terminal cysteine was labeled using maleimide chemistry) was purchased from ThermoFisher and solved in PBS containing 1 mM DTT and 10% glycerol at a final concentration of 200 µM. Single-use Aliquots were snap-frozen and stored at -80°C. For STED imaging, the non-fluorogenic Lifeact-AF594 was diluted to a final concentration of 1 µM in 100 mM tris buffer pH 8.0 containing 5% glucose, 0.5 mg/ml glucose oxidase and 40 µg/ml catalase (all components were purchased from Sigma).

Live cell imaging

HeLa cells were plated on Mattek chambers **(**#P35G-1.5-10-C) 24-48h before imaging. SiR-Tubulin and SiR-lysosome (Spirochrome, Switzerland) staining was performed at 1µM concentration for 1h in DMEM cell culture medium at 37°C (according to the manufacturer's recommendations, stock solution in DMSO diluted 1:1000). Cells were washed twice to remove excess SiR-lysosome or SiR-tubulin labels. Imaging was performed in Imaging Medium consisting of Minimum Essential Medium Eagle (Sigma) and 30 mM HEPES pH 7.4 (Biomol) supplemented with 300 nM Nile Red (dissolved in MeOH and diluted 1:334) at room temperature. During long-term time-lapse imaging, the frame acquisition time was kept shorter than the cycle time to allow for label exchange and reduce photo-stress. Detailed information about imaging parameters for live-cell measurements are listed in Supplementary Table 1.

For phototoxicity control experiments, HeLa Kyoto cells were seeded in CELLview Slides (Greiner Bio-One, catalogue number 543079) and sealed with a gas-permeant, but water impermanent membrane (#4ti-0516/96, 4titude, Wotton, UK) to prevent evaporation of the imaging medium (Phenol-red free DMEM containing 4.5 g/l glucose, 5% FBS and 1% GlutaMAX). Imaging was performed at 37°C and 5% CO_2. 10-11 positions were selected and an overview CLSM image was recorded for each position. Afterwards, 25 STED images were recorded while cycling through all predefined positions and recording one STED image per position and cycle. This resulted in one STED image every 2 min for each position. After STED acquisition, CLSM image stacks (5 slices, 3.75 µm spacing) were taken every 30 min at lower resolution and zoom to follow the cells reliably during cell division for >24h at minimal additional light dose. The experiment was performed in triplicate.

Image analysis

Image stacks were exported in TIFF format and further processed using the open-source image analysis package Fiji (v1.51w). Image stacks of fixed cells (z-stacks or multi-frame measurements) were aligned using the Fiji plugin "Stackreg" (translation). For the determination of intensity time traces shown in Figure 1b and Figure S1, regions of interest were manually selected around single bacteria and the integrated intensity of each bacterium was measured. The raw data was then further processed using OriginPro 2018 (OriginLabs). Therefore, intensity traces were normalized (divided by maximum value) and subsequently averaged for all measured cells. For Nile Red, 3 (high intensity) or 9 cells (low intensity) were analyzed, while 3 (high intensity) or 7 cells (low intensity) contributed to the WGA-AF594 intensity time traces. For JF_{646} azide bleaching curves, 10 (high intensity) or 16 cells (low intensity) were analyzed and 3 (high intensity) or 10 cells (low intensity) contributed to JF_{646}-Hoechst intensity time traces. Images and image series with visible instable focus (drift) were removed from the analysis.

For Figures 2 except Figure 2d and Supplementary Videos 2-6, background was subtracted using the Fiji plugin "Subtract Background" with a rolling ball radius of 25-50 px (see Supplementary Table 1). Parameters were kept identical for CLSM and STED images.

Image deconvolution was carried out using Huygens Professional version 16.10.1p2 (Scientific Volume Imaging B.V.) and applied parameters are summarized in Supplementary Table 1.

For the analysis of (photo)toxicity experiments, STED-imaged cells and 1-2 control cells (randomly chosen) were selected in the initially recorded overview CLSM image. Maximum-intensity projections of the time-lapse confocal z-stacks were created using the plugin "Z Project" in Fiji. Marked cells were traced over the duration of the CLSM time-lapse image sequence in order to count the number of cells that divided normally, divided with division defects (resulting in multinucleated cells), did not divide or died within 24 h after STED imaging.

Supplementary Figures

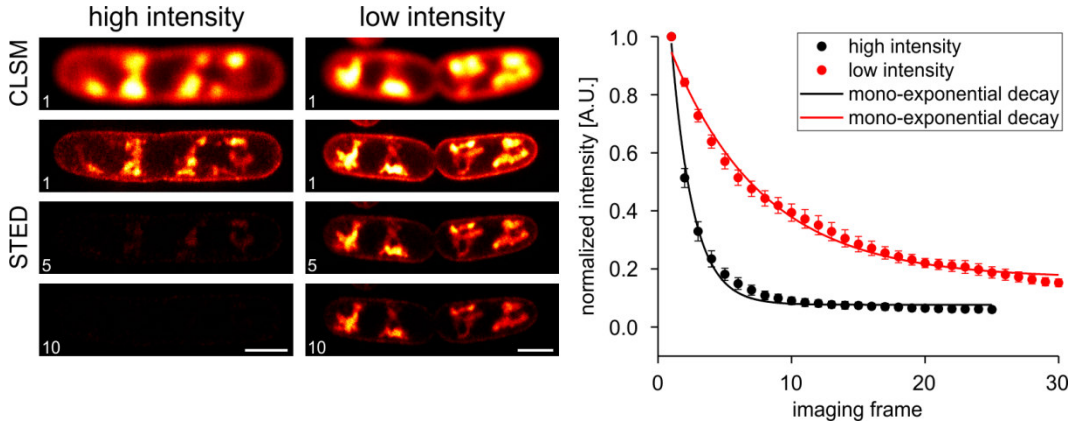

Figure S1: Multi-frame STED imaging of *E. coli* cells stained for chromosomal DNA using ATTO647N. Photobleaching curves were extracted from imaging data recorded with two different settings, as shown by the representative images (left). Numbers indicate the imaging frame. Note that ATTO647N azide has an increased affinity to the E. coli membrane compared to JF_{646}-Hoechst azide (Figure 1). Data points represent mean values from 5 (high intensity) or 9 (low intensity) individual cells and error bars the respective standard deviation. The resulting bleaching curves could be fitted using an exponential decay fit function (red circles: 50% depletion laser power, black circles: maximum depletion laser power; varying excitation laser powers for the different labels, see Supplementary Table 1). Scale bars are 1 µm.

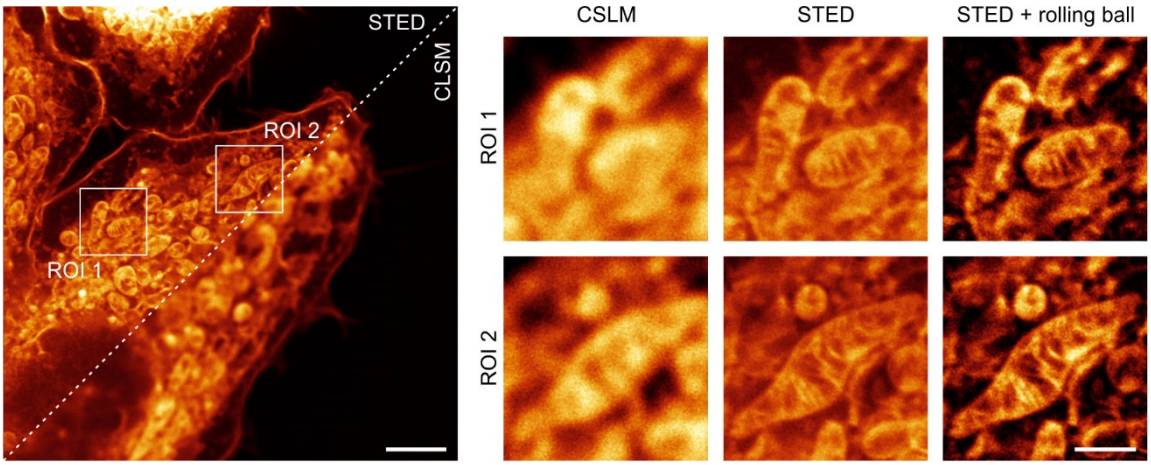

Fig S2: STED image of cellular membranes stained with the exchangeable fluorophore Nile Red. The left image shows a HeLa cell stained with Nile Red and recorded both in CLSM and STED imaging mode. White rectangles indicate the magnified regions shown in the right image panel. STED imaging reveals the internal cristae membranes of intact mitochondria, which are not visible in the respective CLSM images. Image contrast was improved by background subtraction using a rolling ball algorithm in Fiji (50 pixel radius). Scale bars are 3 µm (overview image) and 1 µm (magnified ROIs).

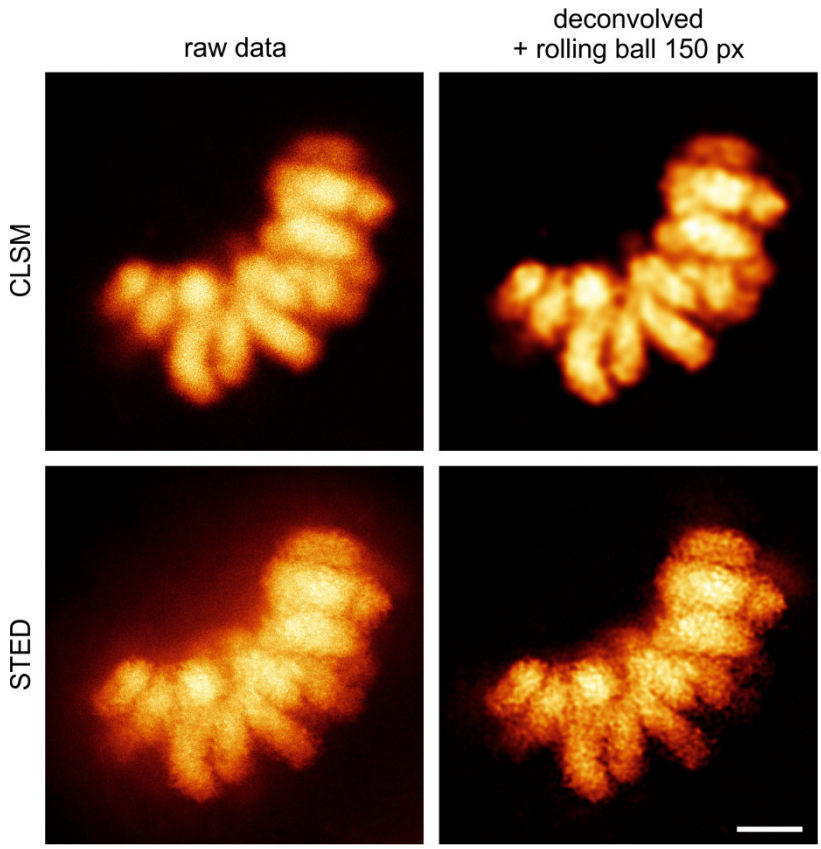

Figure S3: Image processing in CLSM and STED images of mitotic chromosomes shown in Figure 2b. 2D STED image of a HeLa cell chromosome stained with JF_{646}-Hoechst. The grained structure of mitotic chromosomes is visible in the STED image, which is not resolved in the CLSM. Deconvolution and subsequent background subtraction (rolling ball with a radius of 150 px) improved image contrast. Scale bar is 2 µm.

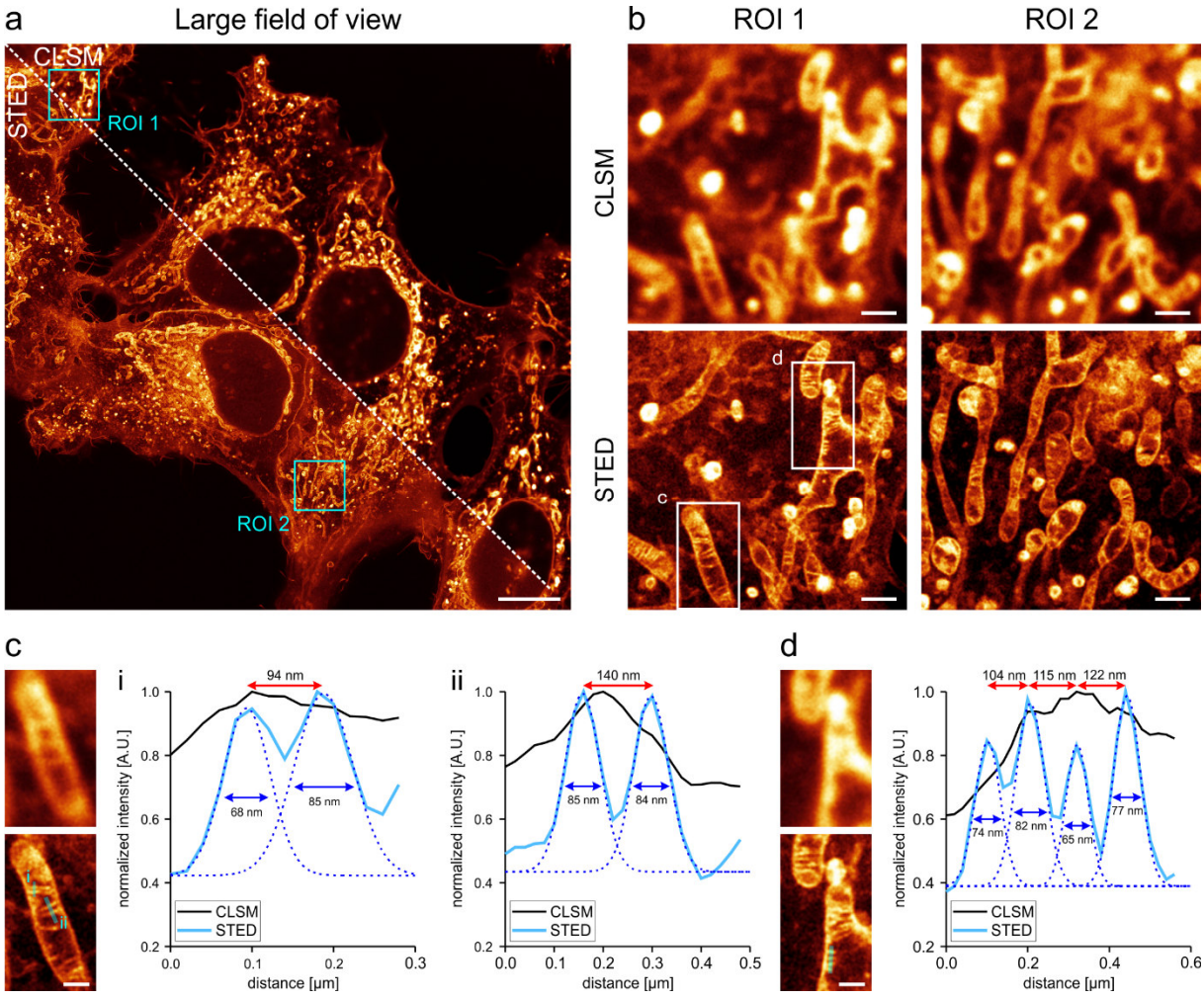

Figure S4: STED imaging of fixed HeLa cells (4% FA) using 1 µM FM4-64. (a) Comparison of CLSM and STED images in a large region of interest. (b) Magnified regions marked by the cyan rectangles in (a). Mitochondria morphologies can only be deduced in the CLSM images (top panel). STED images (bottom panel) show the detailed mitochondrial morphology including internal cristae structures. (c, d) Close-ups of individual mitochondria marked in (b). The strong increase in resolution compared to the CLSM images (top) is clearly visible in the STED images (bottom). Intensity profiles (60 nm line width) were measured along the lines indicated in the STED images (cyan). Cristae widths (FWHM) and peak distances were determined using multi-component Gaussian fit functions. Scale bars are 10 µm in (a), 1 µm in (b) and 500 nm in (c) and (d). A smooth filter was applied to the ROIs in Fiji.

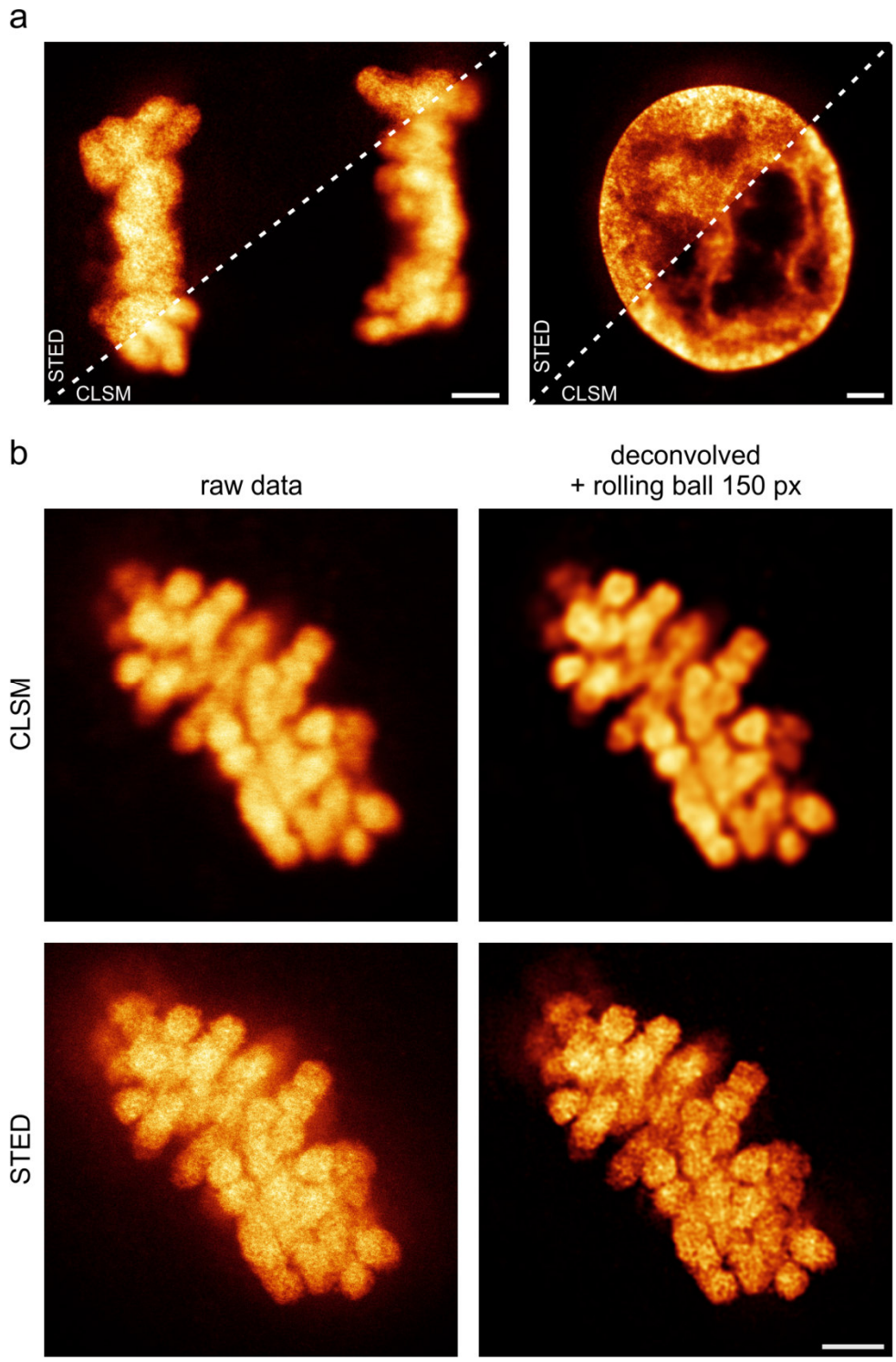

Figure S5: Exchange-based STED imaging of HeLa chromatin using SiR-Hoechst. 300 nM SiR-Hoechst in 150 mM tris pH 8.0 was added to fixed (4% FA) HeLa cells. (a) Comparison of CLSM with STED imaging for a mitotic (left image) and interphase cell (right image). A rolling ball background subtraction (radius 150 pixel) and smooth filter was applied both for CLSM and STED images. For the interphase nucleus on the right, 10 images were averaged for both imaging modes. No deconvolution was applied. (b) Comparison of raw and post-processed data according to Figure S2. Scale bars are 2 µm.

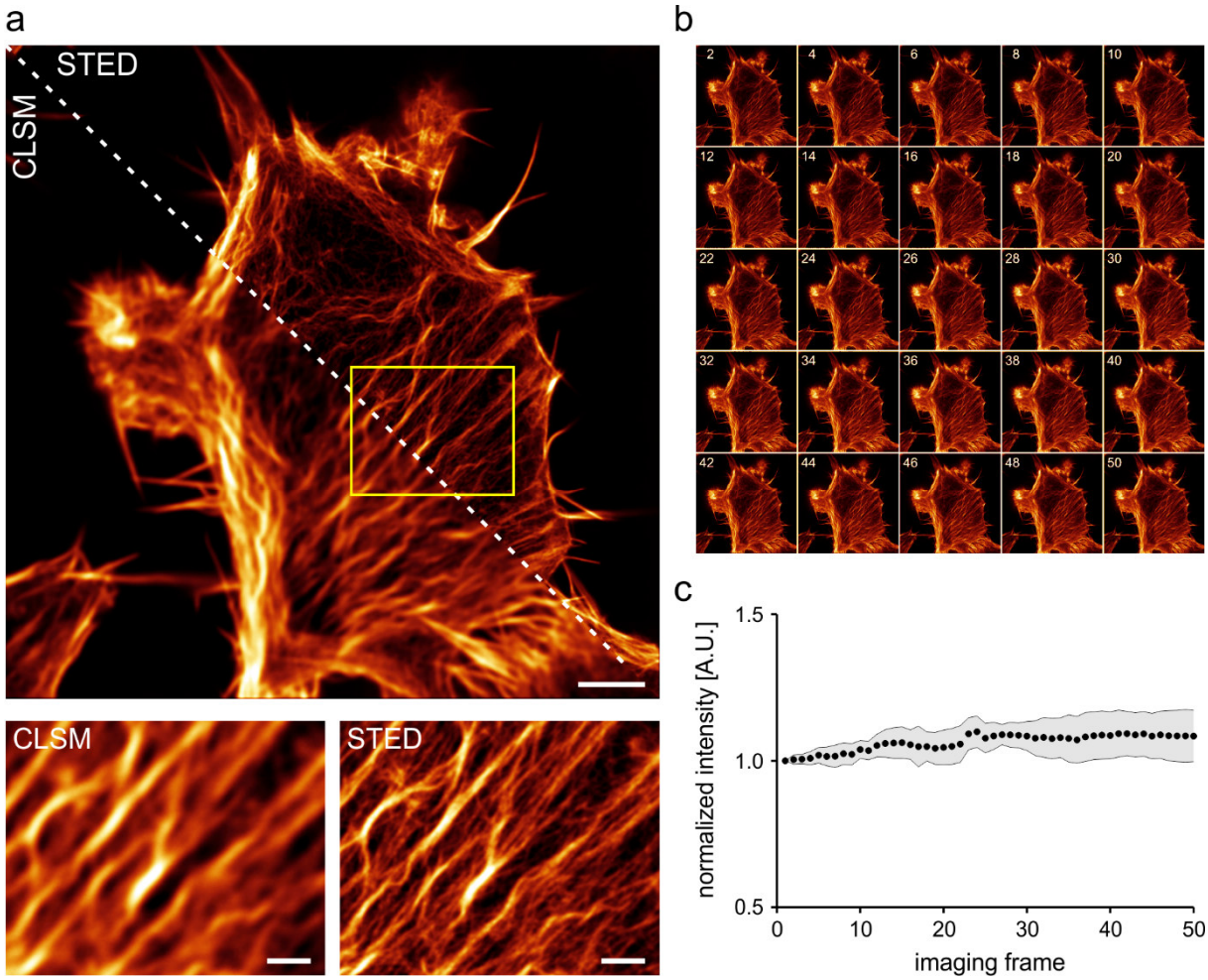

Figure S6: STED imaging of the actin cytoskeleton in fixed HeLa cells labeled with the IRIS probe Lifeact-AF594. (a) Comparison of CLSM and STED average images, created from 50 successive imaging frames acquired using 1 µM Lifeact-AF594. The bottom panel shows the magnified region indicated in the overview image. (b) Montage of the STED time series used for the average image shown in (a). Every second frame of the 50 frame time series is shown. The constant label exchange provided by the Lifeact peptide enables long-time imaging independent of photobleaching. (c) Averaged intensity time trace of 3 independent measurements from two sets of samples. No bleaching is observed over the whole measurement time course. Data points represent mean values, black lines and shaded area the respective standard deviation. Scale bars in (a) are 3 µm (overview) and 1 µm (inset).

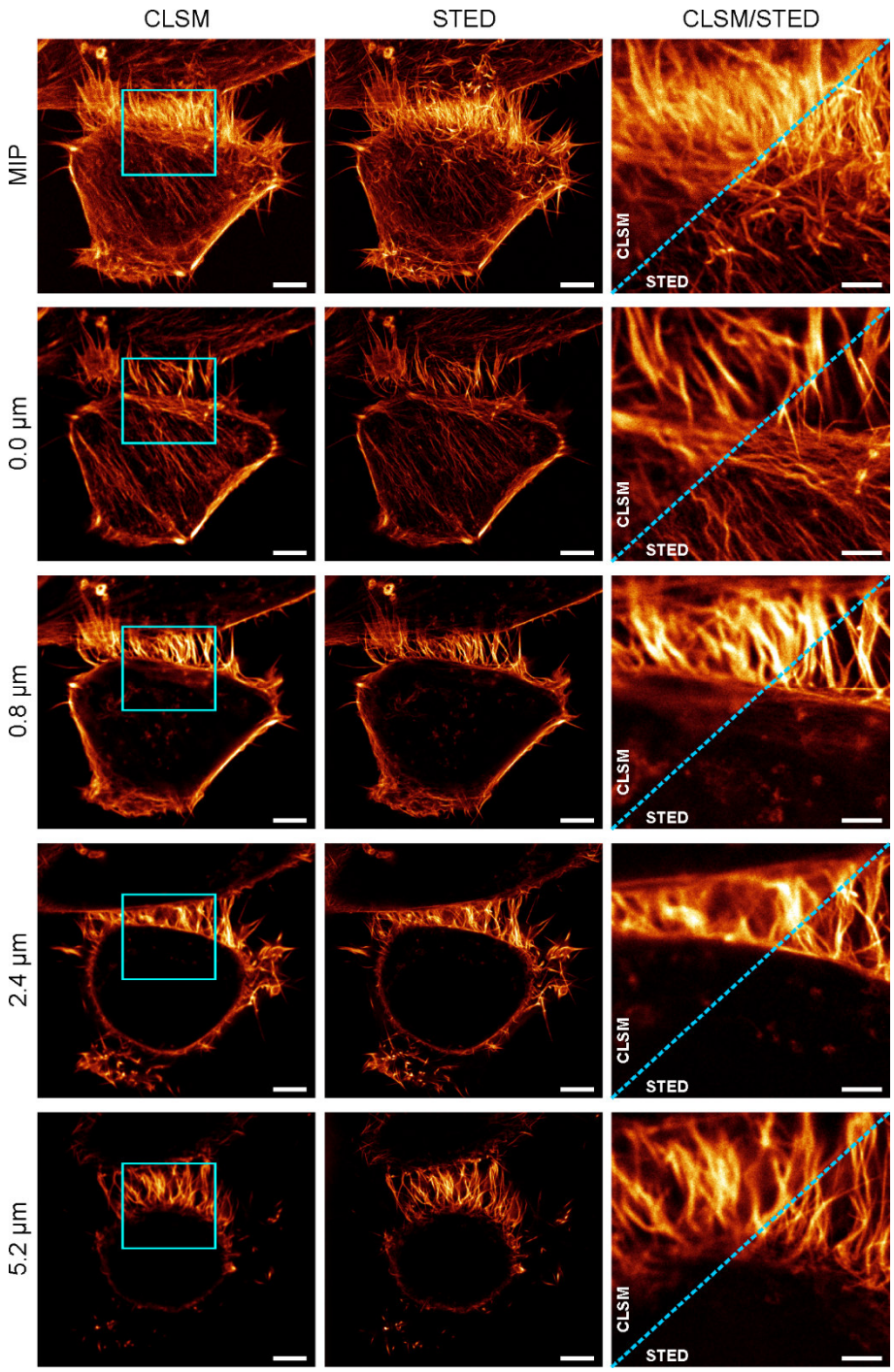

Figure S7: Whole cell exchange-based STED image of a fixed HeLa cell. The z-stack was recorded using 1 µM Lifeact-AF594 with a spacing of 400 nm. Only selected frames are shown. The upper image panel shows the maximum intensity projection (MIP) of the entire image stack, both for in CLSM (left image column) and STED imaging mode (mid image column). The right image column shows a direct comparison of the magnified region indicated in the CLSM images. Brightness and contrast were kept identical for all imaging planes to maintain comparability. The numbers indicate the axial focus position. Scale bars are 5 µm for the large field of view and 2 µm for the magnified region.

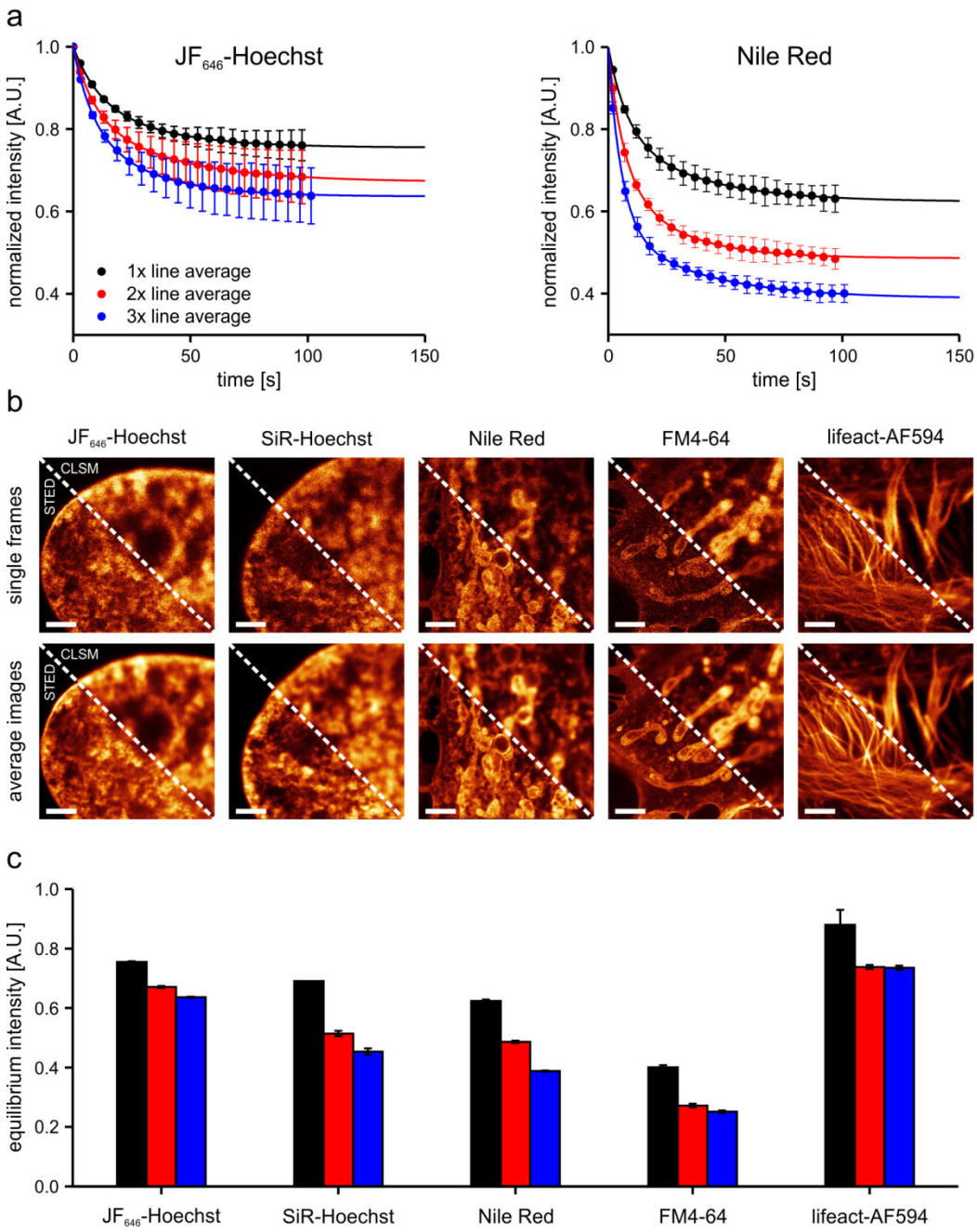

Figure S8: Fast time-lapse STED imaging using exchangeable fluorescent probes. (a) Normalized intensity time traces of JF_{646}-Hoechst (left) and Nile Red (right) using different imaging parameters. Time series were recorded at ~1 s interval exhibiting a varying exposure to light depending on the line average set (0.356, 0.712 and 1.04 s for 1x, 2x and 3x line averaging, respectively). For visualization, 5 imaging frames were binned and time traces were fitted using a bi-exponential decay fit function. The intensity approaches a plateau, representing the equilibrium between the bleaching, binding and unbinding rates of the label used. Higher number of line averages increases the bleaching rate which results in lower equilibrium intensities. (b) Representative CLSM and STED images for the labels used in this study. Shown are either single imaging frames (upper panel) or average images (16x line average for CLSM, 30-50 frames averaged for STED images). (c) Analysis of the equilibrium intensities derived from the bi-exponential fits as shown in (a). Under the conditions applied, JF_{646}-

Hoechst and Nile Red exhibit higher equilibrium intensities compared to SiR-Hoechst and FM4-64. Overall, the IRIS probe Lifeact-AF594 provides the highest equilibrium intensities. Data points in (a) and bars in (c) represent mean values from 3-4 independent measurements and error bars the respective standard deviations. Label were added at 300 pM concentration, except for FM4-64 and Lifeact-AF594 (1 µM). Scale bars in (c) are 2 µm.

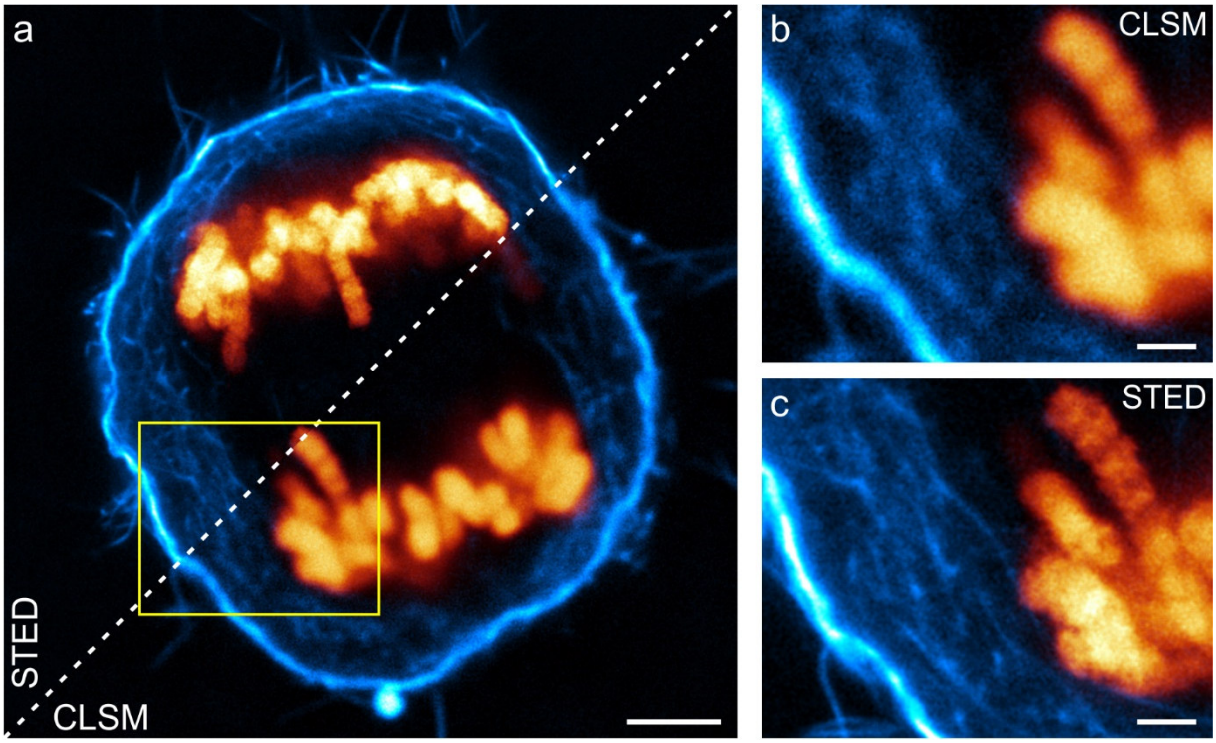

Figure S9: Dual-color STED image of a mitotic HeLa cells recorded using Lifeact-AF594 and JF_{646}-Hoechst. (a) Comparison of CLSM and STED images showing the actin cytoskeleton (cyan) and condensed chromosomes (orange to white). (b) Magnified region of the dual-color CLSM and (c) STED image indicated by the yellow rectangle in (a). The cortical actin and chromosomes are resolved at much greater detail in the STED image. Scale bars are 3 µm in (a) and 1 µm in (b) and (c).

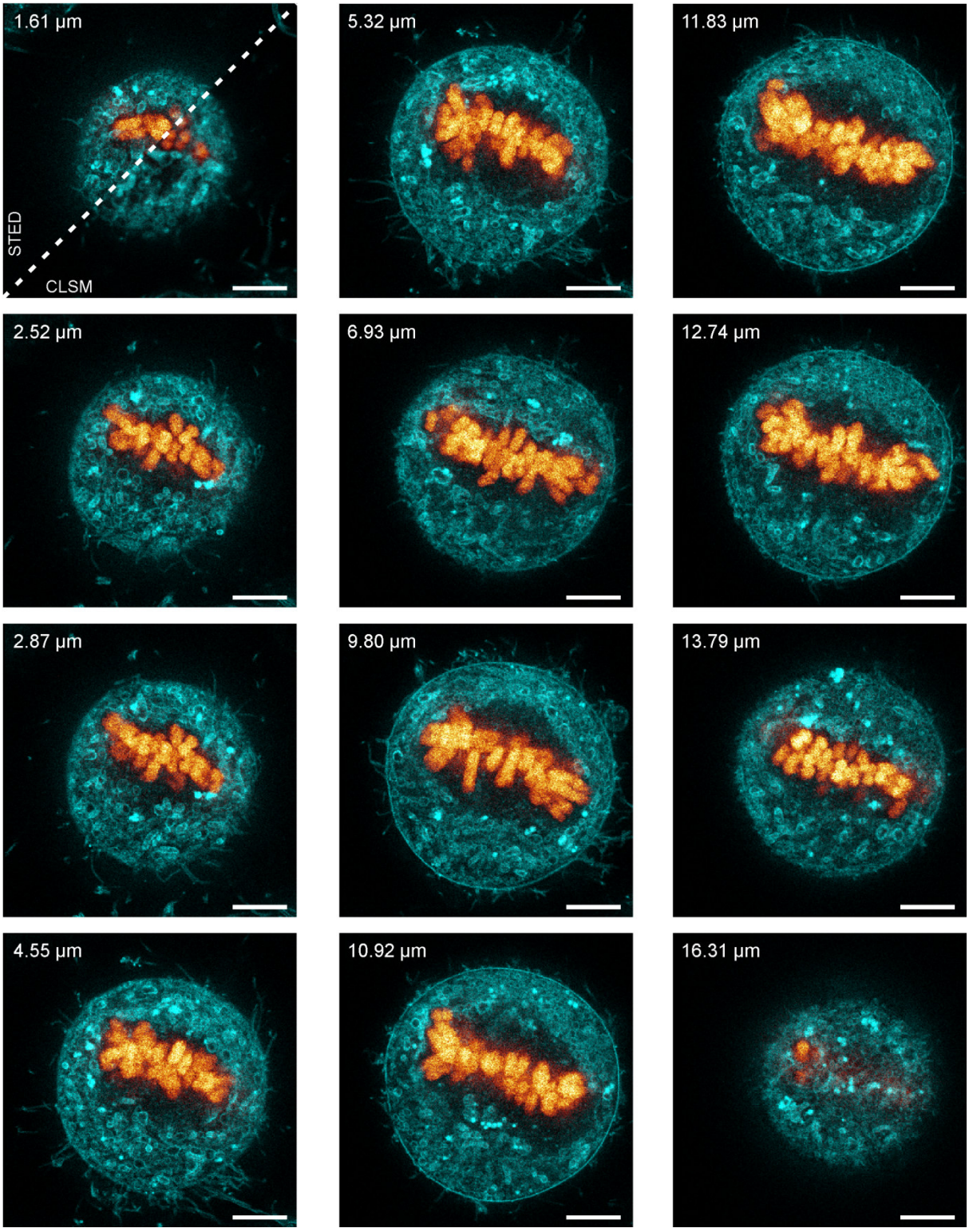

Figure S10: Dual-color STED images of a fixed HeLa cell during mitosis. Exchangeable fluorophores (Nile Red (cyan), JF_{646}-Hoechst (glow)) facilitate whole-cell imaging with a constant fluorescent signal in all images. Brightness and contrast were kept identical for all imaging planes to maintain comparability. The numbers indicate the axial focus position. Note that only representative positions are shown and that the axial stepping size conducted 70 nm (see Supplementary Table 1). Scale bars are 5 µm.

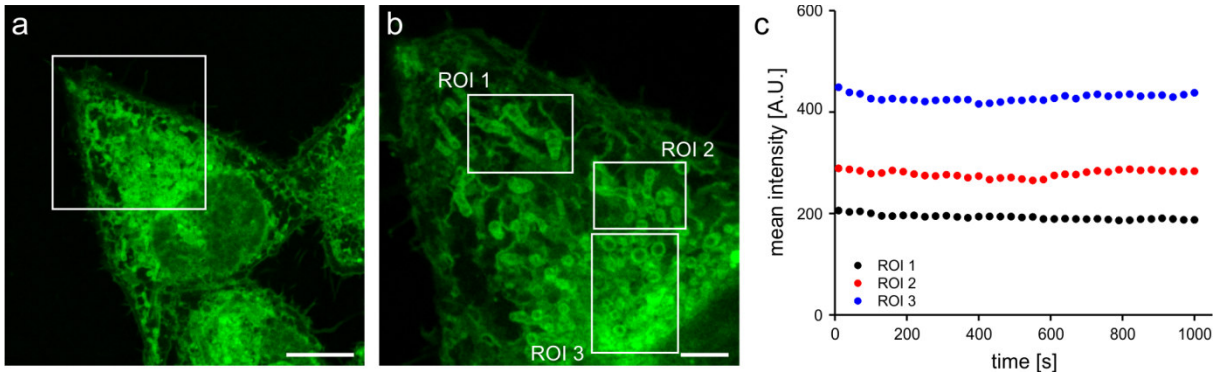

Figure S11: Photobleaching-independent live cell STED imaging with the exchangeable fluorophore Nile Red. (a) CLSM image of a HeLa cell stained with Nile Red. (b) STED image of the region indicated in (a) (white rectangle). White rectangles in (b) represent ROIs used for intensity analysis. (c) Nile red signal during time-lapse STED imaging. The fluorescence signal is constant over ~ 16 min of STED imaging. The time-lapse video is provided as Supplementary Video 4. Scale bars are 10 µm (a) and 3 µm (b).

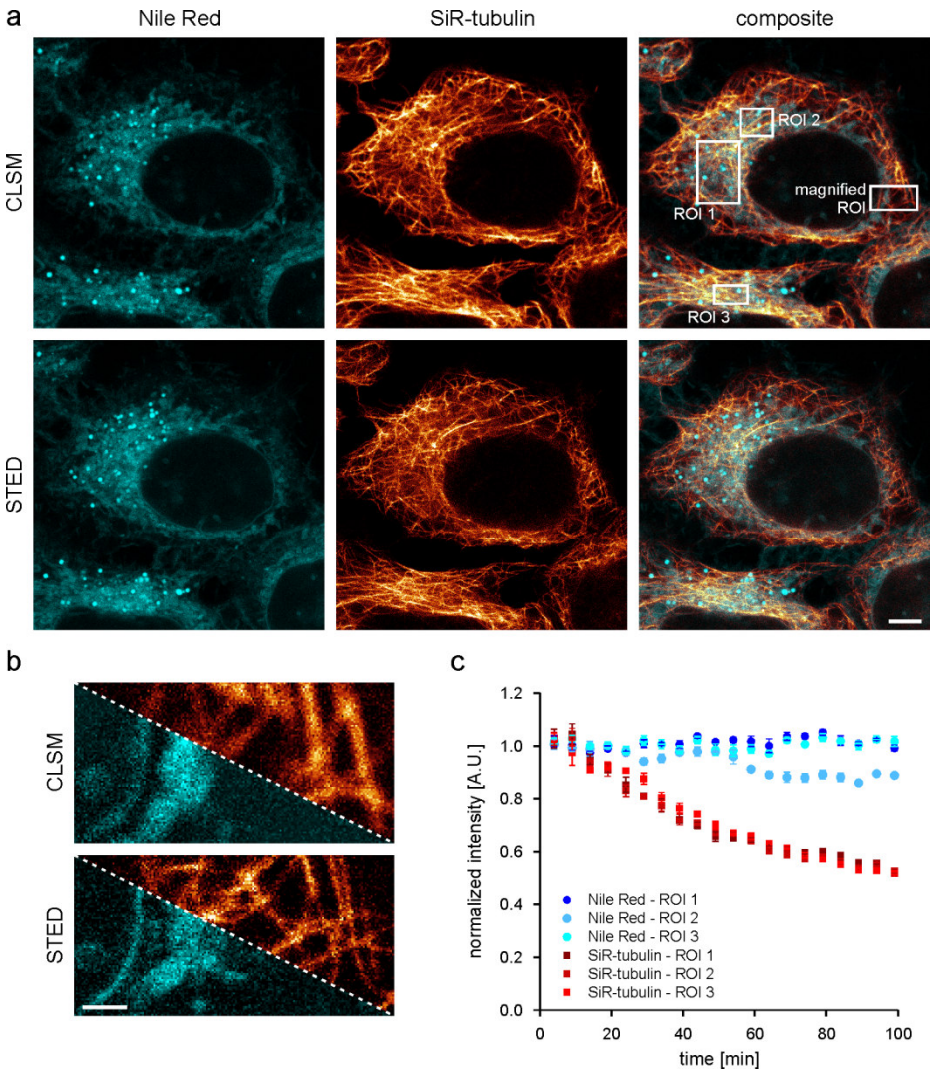

Figure S12: Dual-color live-cell STED microscopy with exchangeable and 'static' labels. (a) HeLa cells were stained for membranes (Nile Red, cyan) and microtubules (SiR-Tubulin, glow). (b) Magnified ROI of (a), as indicated by the white rectangle. The applied imaging conditions provide an enhanced resolution for both labels using a single depletion laser (775 nm). (c) Fluorescence intensity over time, extracted from ROIs shown in (a) (white rectangles) for both Nile Red and SiR-Tubulin. Photobleaching is observed for the static label SiR-Tubulin, while the exchangeable label Nile Red shows an almost constant signal for ~ 100 min. Data points represent mean values obtained by averaging of 5 consecutive imaging frames and error bars the respective standard deviations. The time-lapse video is provided as Supplementary Video 7. Scale bars are 5 µm (a) and 1 µm (b).

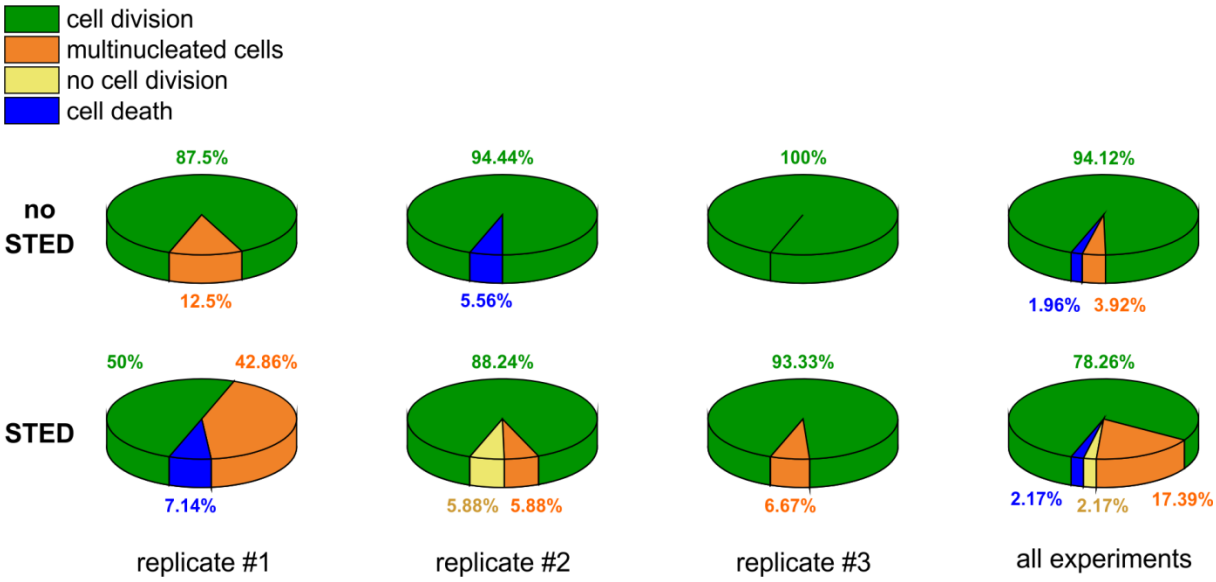

Figure S13: Quantification of potential (photo)toxic effects during live-cell STED imaging using Nile Red. Imaging buffer contained 300 nM Nile Red for the entire experiment. Imaging and image analysis was performed as described in the methods section. For cells solely imaged using CLSM (upper pie charts), 94% of all randomly selected cells divided within 24 h, indicating that Nile Red and CLSM imaging do not induce severe toxic effects. After live-cell STED imaging, the majority of cells divided normally. However, an increased fraction of multinucleated could be observed for cells exposed to STED. Note that most multinucleated cells, both for STED-imaged and control cells, were observed in one replicate. This indicates that cells might have been stressed prior to the imaging procedure making the more susceptible to phototoxic effects. At least 14 cells were selected for each imaging mode and replicate. In total, n = 54 (control) and 46 cells (STED).