Bleaching-independent, 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% CO2 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, HeLa Kyoto cells used for live cell imaging 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 Scientific) 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% TX-100 (Sigma) in PBS for 40 min and subsequently rinsed 2x with PBS.

Click-labeling of nascent *E. coli* DNA using JF_{646} 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 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.

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 was signal accumulated.

JF_{646}-Hoechst and Nile Red were diluted in 150 mM tris pH 8.0 (ThermoFisher) to stock solutions of 100 µM in DMSO or methanol, respectively. For imaging, a final concentration of typically 300 nM was used.

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 for 1h in DMEM cell culture medium at 37°C (according to the manufacturer's recommendations, stock solution in DMSO diluted 1:1000). Imaging was performed in Imaging Medium consisting of Minimum Essential Medium Eagle (Sigma) and 30 mM HEPES pH 7.4 (Biomol) supplemented with 400 nM Nile Red (dissolved in MeOH and diluted 1:334) at room temperature. During 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.

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 (divide 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 2b-e 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.

Supplementary Figures

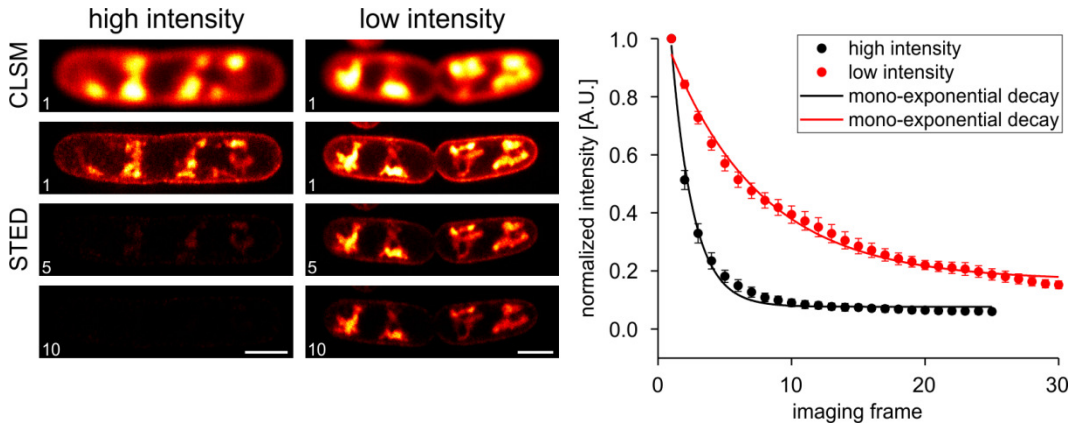

Figure S1: Multi-frame STED imaging of *E. coli* cells stained for chromosomal DNA using ATTO647N. Photobleaching curves were extracted from imaging data recorded with two different settings, as shown by the representative images (left). Numbers indicate the imaging frame. Note that ATTO647N azide has 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. Scale bars are 1 µm.

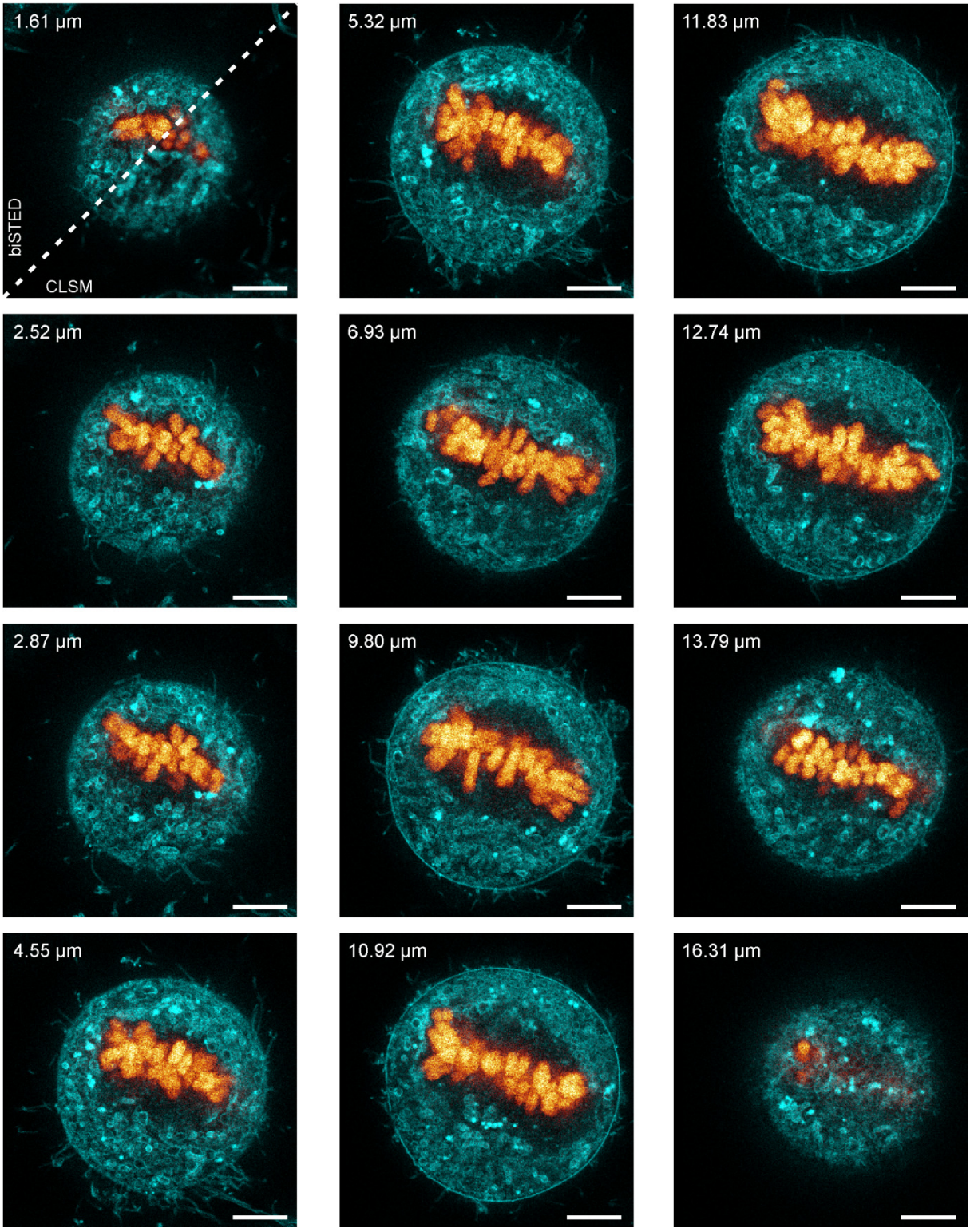

Figure S2: Dual-color biSTED images of a fixed HeLa cell during mitosis. Exchangeable fluorophores (Nile Red (cyan), JF_{646}-Hoechst (glow)) facilitate whole-cell imaging with a constant fluorescent signal in all images.. Brightness and contrast were kept identical for all imaging planes to maintain 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.

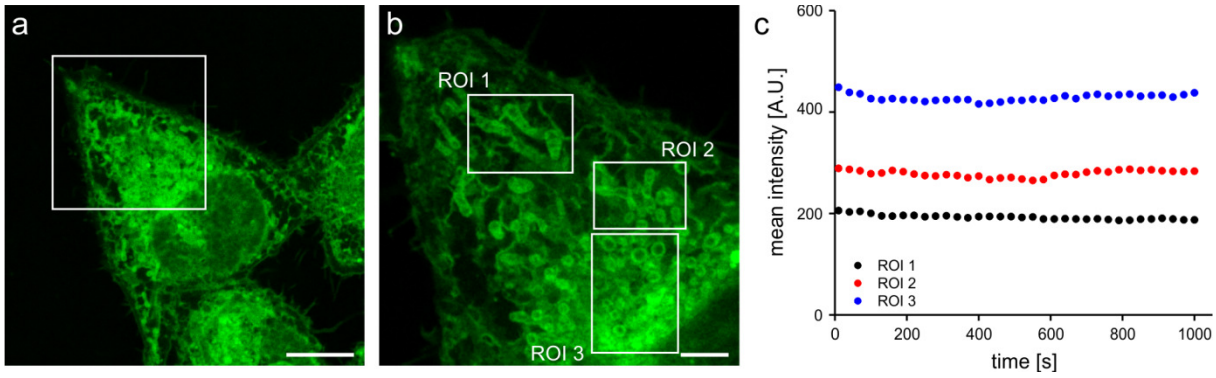

Figure S3: Photobleaching-independent live cell STED imaging with the exchangeable fluorophore Nile Red. (a) CLSM image of a HeLa cell stained with Nile Red. (b) STED image of the region indicated in (a) (white rectangle). White rectangles in (b) represent ROIs used for intensity analysis. (c) Nile red 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 (a).

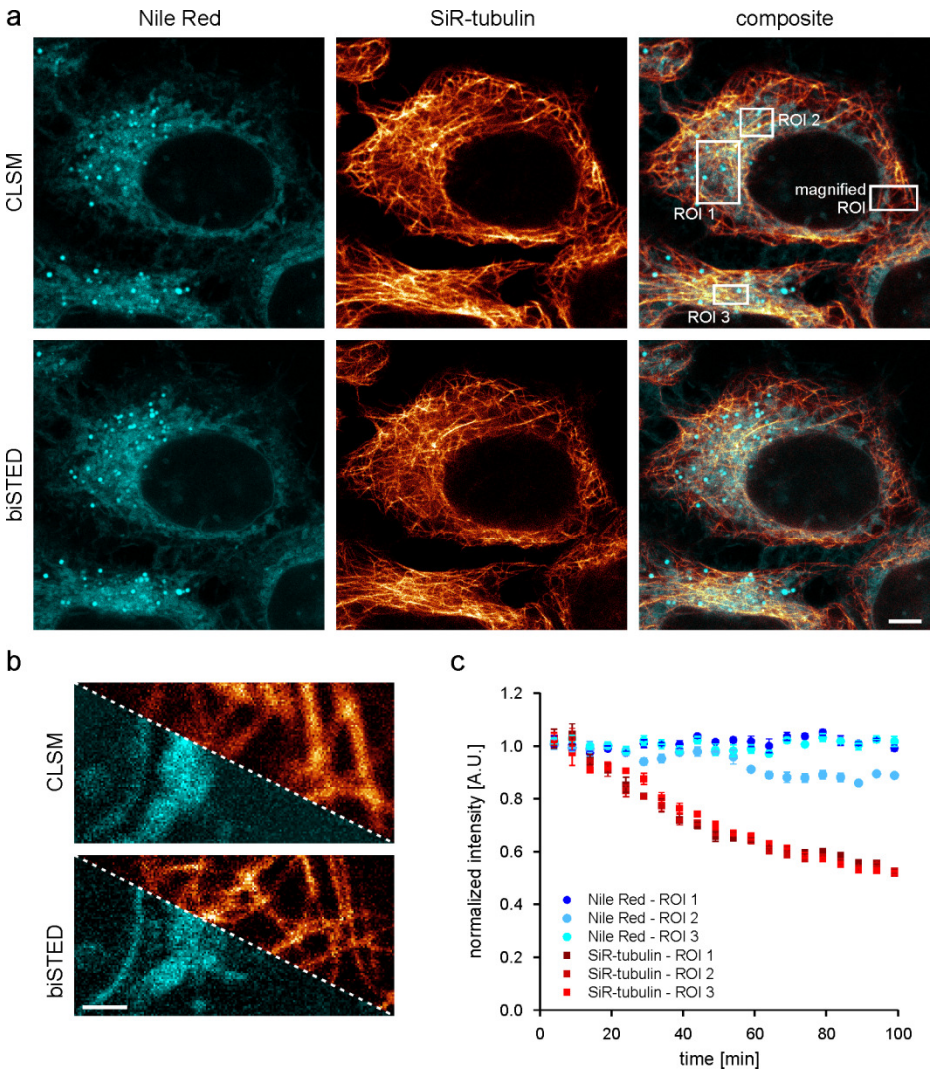

Figure S4: Dual-color live-cell STED microscopy with exchangeable and 'static' labels. (a) HeLa cells were stained for membranes (Nile Red, cyan) and microtubules (SiR-Tubulin, glow). (b) Magnified ROI of (a), as indicated by the white rectangle. The applied imaging conditions provide an enhanced resolution 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).

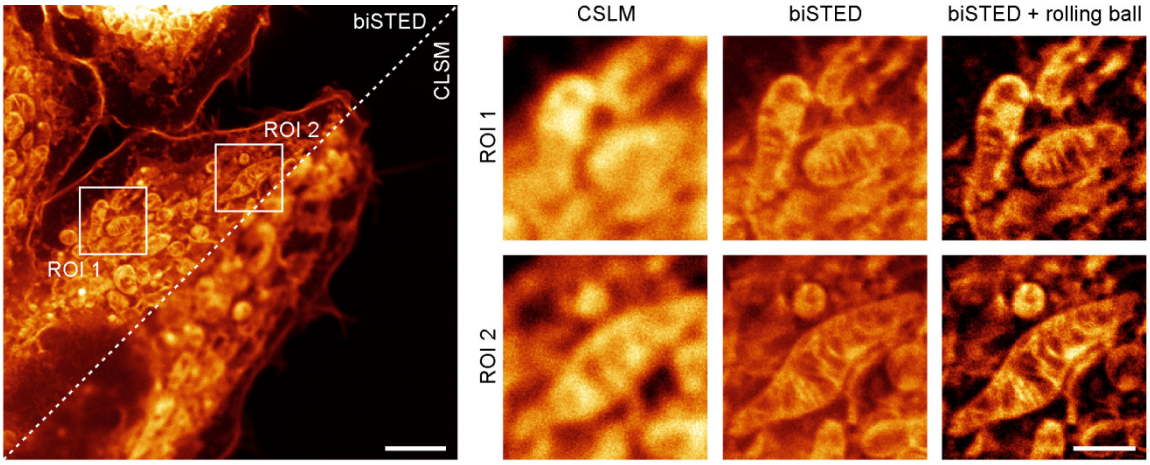

Fig S5: STED image of cellular membranes stained with the exchangeable fluorophore Nile Red. The left image shows a HeLa cell stained with Nile Red and recorded with both biSTED and CLSM. White rectangles indicate the magnified regions shown in the right image panel. STED imaging reveals the internal 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).

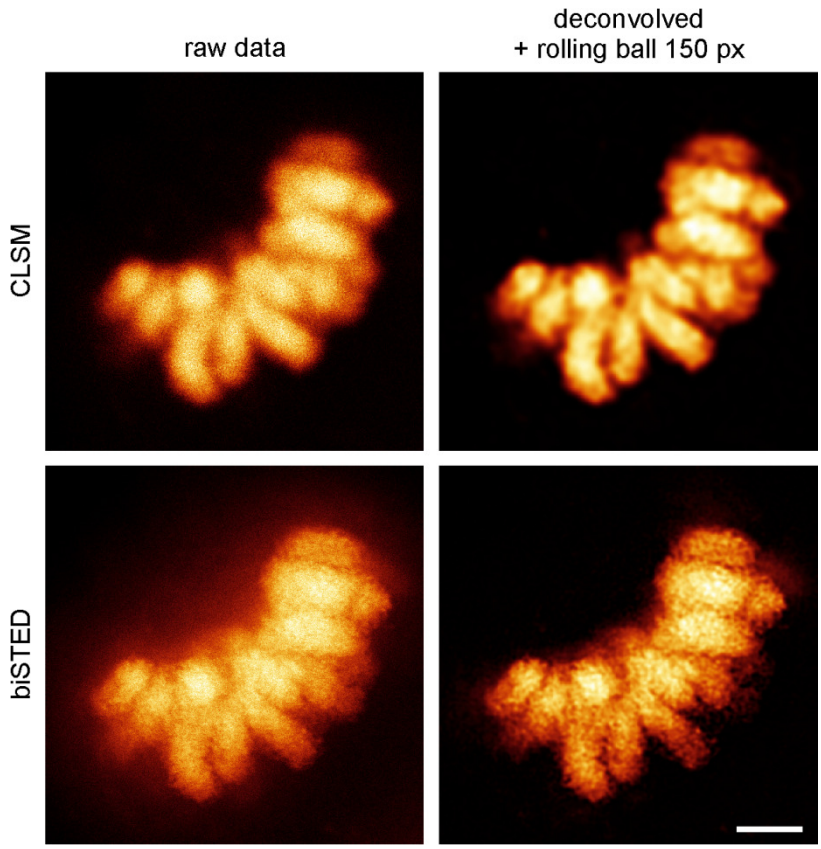

Figure S6: Image processing in CLSM and STED images of mitotic chromosomes shown in Figure 2e. 2D biSTED image of a HeLa cell chromosome stained with JF_{646}-Hoechst. The grained structure of mitotic chromosomes is visible in the biSTED image, which is not resolved in the CLSM. Deconvolution and subsequent background subtraction (rolling ball with a radius of 150 px) improved image contrast. Scale bar is 2 µm.