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Assessing photodamage in live-cell STED microscopy

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To the Editor:

The recent breakthroughs in the development of optical nanoscopy provide unprecedented views of the inner workings of cells. STED (stimulated emission depletion) microscopy, in particular, enables real-time observation of living cells at or below 50 nm resolution[1,2]. However, the high irradiation intensities used in STED nanoscopy have raised concerns about the validity of live-cell observations using this and similar approaches[3,4]. We report here that, under the right conditions, living cells can be imaged by STED nanoscopy without substantial photodamage.

We chose the cytoplasmic level of the divalent cation calcium (Ca^{2+}) as an indicator of cell stress due to its important role at the earliest stages of various cell-death modalities (Supplementary Note 1)[5]. HeLa and COS7 cells were transiently transfected with the SNAP-tagged β-subunit of the endoplasmic reticulum (ER) membrane-localized protein Sec61β. Cells were then labeled with the organic cell-permeable dye SiR-BG, incubated with the Ca^{2+}-sensitive dye FluoForte and irradiated using typical STED imaging conditions[1] with an 8-kHz resonant scanner for about 10 min while monitoring the FluoForte signal (Fig. 1a–c, Supplementary Methods, Supplementary Note 2). Only a minor fraction of cells (3 of 30 HeLa cells; 0 of 30 COS7 cells) (Fig. 1c) showed a stress response distinguishable from non-STED irradiated cells (not statistically different: HeLa p=0.29, COS7 p=1). This response was further reduced, down to a level where all cells showed Ca^{2+}-responses comparable to those observed under non-STED imaging conditions, by applying a reactive

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AUTHOR CONTRIBUTIONS

N.K., A.G. and J.B. conceived and designed the experiments. N.K., A.G. and M.D.L. performed the experiments. All authors analyzed and discussed the data. G.H. performed the statistical analysis. N.K., A.G. and J.B. wrote the manuscript.

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oxygen species (ROS) scavenging buffer (Fig. 1d, Supplementary Methods and Supplementary Note 3). Cells also appeared completely normal in ER morphology and cell shape over the about 10-min time course of STED imaging (Fig. 1e–l, Supplementary Note 4, Supplementary Video 1).

We could observe, however, that using a slower scanner (1 kHz) led to a more pronounced FluoForte response, which suggests that concentrating the irradiation of an area in time rather than distributing it more evenly increases photodamage (Supplementary Note 5). Interestingly, the stress response also depended on which cellular compartment – ER, mitochondria (outer membrane protein 25, OMP25), Golgi (α-mannosidase II, ManII) or histones (H2B) - was labeled (Supplementary Methods, Supplementary Note 6) and how much SiR dye was present in each cell (Supplementary Note 7). The latter observations suggest that stress was mediated through light absorption of the SiR dye itself.

Based on our experimental results and literature research we recommend the following guidelines (arranged by workflow) to minimize photodamage in STED nanoscopy:

- Minimize pre-imaging stress of cells; e.g. consider using electroporation instead of transfection reagents (Supplementary Note 8).
- Limit overexpression of tag proteins (e.g. SNAP) and titrate the amount of fluorescent dye (e.g. SiR-BG).
- Perform experiments on the microscope under optimal cell culture conditions (temperature, CO_2, osmolarity, and minimal mechanical stress).
- Consider using ROS scavenging buffer; we recommend a variation of two previously published buffers (Supplementary Methods)[6,7].
- Use far-red depletion and excitation wavelengths (Supplementary Note 9)[8].
- Image with a fast resonant scanner (e.g. 8 or 16 kHz).
- Limit laser intensities to values required for the desired resolution (e.g. about 140 mW depletion (775 nm) and about 20 μW excitation power (640 nm) for <50 nm resolution)[1] (Supplementary Note 10).

Our survey focused on the first about 10 min of imaging, a time frame that allows the investigation of a large range of cell biological phenomena. A previous study has shown that long-term (20–24 h) viability of cultured cells is compromised after irradiation doses typical for (F)PALM and (d)STORM[8]. When monitoring cells for 24 h following STED exposure, we could observe an increase in cell death compared to controls (HeLa p=0.021, COS7 p=0.091; Fig. 1m, Supplementary Videos 2 **and** 3, Supplementary Note 11) suggesting that long-term cell health is impaired. It is important to point out, however, that >25% of STED-irradiated cells in these 24-h experiments were undistinguishable from live control cells proving that STED exposure does not lead to certain death. More importantly, the fact that live-cell STED nanoscopy can be performed without inducing substantial short-term damage responses is good news to the cell biology community, which critically depends on nanoscopy methods to resolve dynamics and structures below 50 nm.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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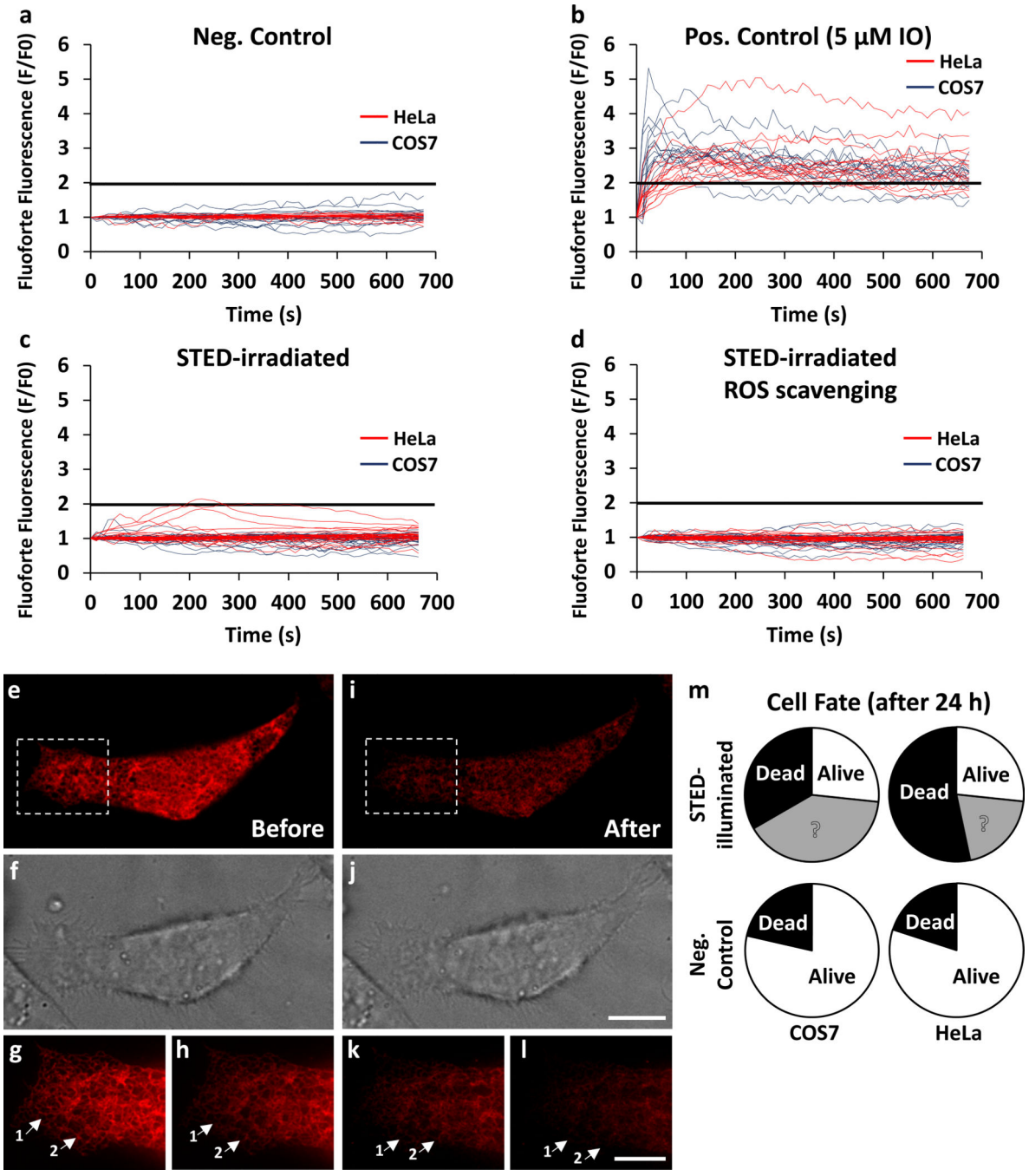

Figure 1 |. Short and long-term effects of live-cell STED imaging on COS7 and HeLa cells.

(a) Cytoplasmic Ca^{2+}-level response of SNAP-Sec61β expressing SiR-labeled cells under negative control conditions (no excitation or STED illumination). **(b)** Positive control using Ionomycin. **(c)** STED-irradiated cells using an 8-kHz resonant scanner. **(d)** STED-irradiated cells with ROS scavenging buffer added. **(e-l)** Representative fluorescence (e,g-i,k,l) and brightfield (f,j) images of a HeLa cell before and after STED irradiation in ROS scavenging buffer visualizing cell viability via cell morphology and ER movement. Scale bars: 10 µm (j), 5 µm (l). Confocal images (e,i), STED images (g,h,k,l). **(m)** Long-term viability of

STED-irradiated and control cells. Cells are categorized in alive, dead and indeterminable (labeled as “?”; see Supplementary Methods) after 24 h. Statistical information (N = total number of cells; M = number of independent experiments): (a) HeLa: N=17, M=3; COS7: N=18, M=4; (b) HeLa: N=15, M=3; COS7: N=15, M=3; (c) HeLa: N=30, M=3; COS7: N=30, M=4; (d) HeLa: N=32, M=4; COS7: N=30, M=5; (e-l) N=10, M=2; (m) HeLa: N=15, M=3; COS7: N=15, M=3; control HeLa: N=20, M=3; COS7: N=28, M=4.