Assessing photodamage in live-cell STED microscopy

To the Editor — The recent breakthroughs in the development of optical nanoscopy have provided unprecedented views of the inner workings of cells. Stimulated emission depletion (STED) microscopy, in particular, allows real-time observation of living cells at resolutions of 50 nm or less[1,2]. However, the high irradiation intensities used in STED nanoscopy have raised concerns about the validity of live-cell observations obtained with 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 because of its important role at the earliest stages of various cell-death modalities[5] (Supplementary Note 1). We transiently transfected HeLa and COS7 cells with the SNAP-tagged β-subunit of the endoplasmic reticulum (ER) membrane-localized protein Sec61β. We then labeled the cells with the organic cell-permeable dye SiR-BG, incubated them with the Ca^{2+}-sensitive dye FluoForte, and irradiated them under 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 that of non-STED-irradiated cells (not statistically different: HeLa, $P = 0.29$; COS7, $P = 1$). Application of a reactive oxygen species (ROS) scavenging buffer reduced this response further, to a level at which all cells showed Ca^{2+} responses similar to those observed under non-STED imaging conditions (Fig. 1d, Supplementary Methods, Supplementary Note 3). Cells also appeared completely normal in ER morphology and cell shape over the ~10-min time course of STED imaging (Fig. 1e–l, Supplementary Note 4, Supplementary Video 1).

We observed, however, that use of 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). The stress response also depended

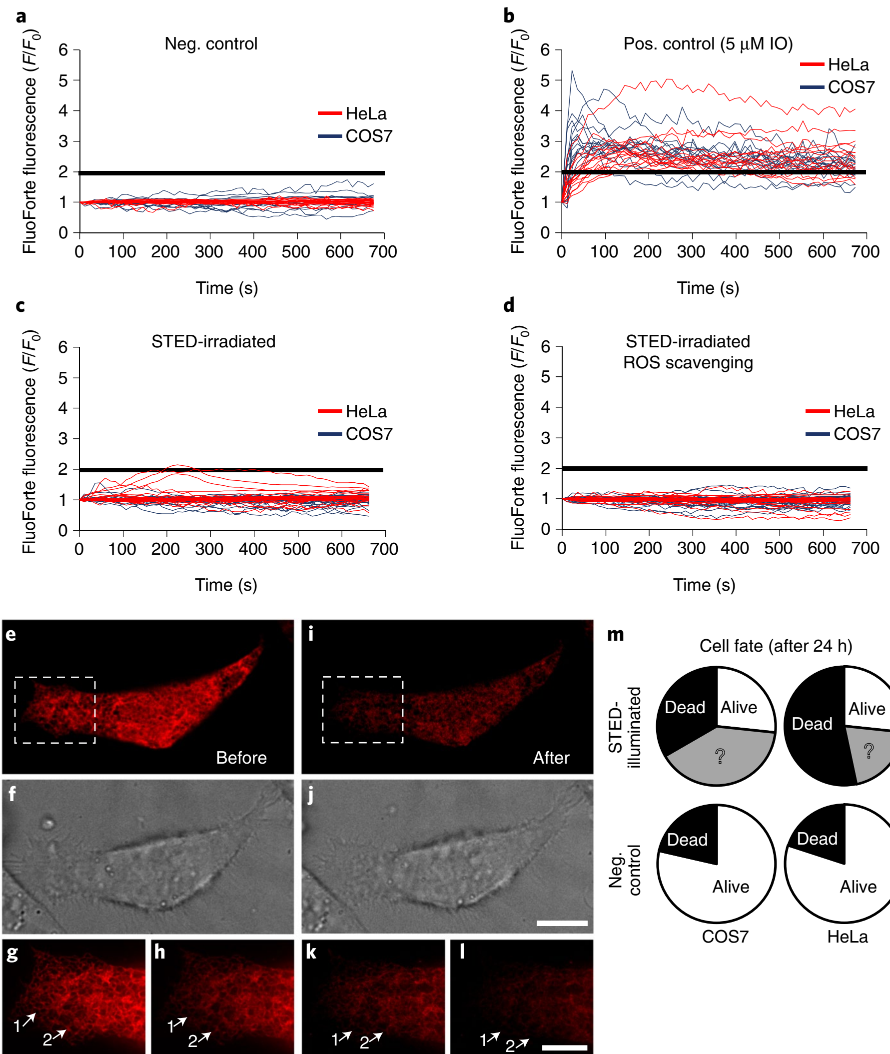

Fig. 1 | Short- and long-term effects of live-cell STED imaging on COS7 and HeLa cells. **a**, Cytoplasmic Ca^{2+}-level response of SNAP–Sec61β-expressing SiR-labeled cells under negative (neg.) control conditions (i.e., no excitation or STED illumination). **b**, Results for the positive (pos.) control with ionomycin (IO) treatment. **c**, Results for STED cells irradiated with an 8-kHz resonant scanner. **d**, Results for STED-irradiated cells with ROS scavenging buffer added. **e–l**, Representative fluorescence (**e**,**g**–**i**,**k**,**l**) and bright-field (**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. Dashed outlines in **e** and **i** indicate the regions shown at higher magnification in **g**,**h** and **k**,**l**, respectively. Scale bars, 10 µm (**e**,**i**,**f**,**j**) or 5 µm (**g**,**h**,**k**,**l**). Confocal images (**e**,**i**) and STED images (**g**,**h**,**k**,**l**) are shown. **m**, Long-term viability of STED-irradiated and control cells. Cells were categorized as alive, dead, or 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, $N = 20$, $M = 3$; control COS7, $N = 28$, $M = 4$.

on which cellular compartment—ER, mitochondria (outer membrane protein 25 (OMP25)), Golgi (α-mannosidase II), or histones (H2B)—was labeled (Supplementary Methods, Supplementary Note 6), and it increased with the amount of SiR dye present in each cell (Supplementary Note 7). These last observations suggest that stress was mediated through light absorption of the SiR dye.

On the basis of our experimental results and the literature, we recommend the following guidelines (arranged by workflow) to minimize photodamage in STED nanoscopy:

- Minimize pre-imaging stress of cells; for example, 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).
- Conduct 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[6,7] (Supplementary Methods).
- Use far-red depletion and excitation wavelengths[8] (Supplementary Note 9).
- 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 ~10 min of imaging, a time frame that allowed the investigation of a large range of cell biological phenomena. A previous study showed that long-term (20–24 h) viability of cultured cells is compromised by irradiation doses typical for (fluorescence) photoactivation localization microscopy and (direct) stochastic optical reconstruction microscopy[8]. When monitoring cells for 24 h after STED exposure, we observed an increase in cell death compared with that in non-imaged 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 was 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, which proves that STED exposure does not lead to certain death. More important, the fact that live-cell STED nanoscopy can be performed without induction of substantial short-term damage responses is good news to the cell biology community, which depends heavily on nanoscopy methods to resolve dynamics and structures below 50 nm.

Reporting Summary

Further information on research design is available in the Nature Research Reporting Summary linked to this article. ❐

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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. N.K., A.G., M.D.L., G.H., D.T., J.E.R., and J.B. analyzed and discussed the data. G.H. performed the statistical analysis. N.K., A.G., and J.B. wrote the manuscript.

Competing interests

J.B. has financial interests in Bruker Corp. and Hamamatsu Photonics. The other authors declare no competing interests.

Additional information

Supplementary information is available for this paper at https://doi.org/10.1038/s41592-018-0145-5.

Mutation frequency is not increased in CRISPR–Cas9-edited mice

To the Editor — CRISPR–Cas9-based genome-editing technologies hold great promise, but the potential for the creation of mutations at nontarget sites could limit their utility. One study reported the identification via whole-genome sequencing (WGS) of hundreds of nontargeted mutations in CRISPR–Cas9-treated mice[1]. Shortcomings of that analysis were its failure to compare parents to progeny, a necessary prerequisite for discrimination of de novo mutations from pre-existing variants in the strain background, and the small number of samples examined (one control and two CRISPR–Cas9-edited animals). As discussed in this journal[2] (Supplementary Table 1), there is a need to understand CRISPR's in vivo genomic effects. To address this, we designed a parent–progeny study (Fig. 1a, Supplementary Methods) and conducted unbiased WGS (Supplementary Table 2) on 6 CRISPR–Cas9-edited mice, 6 control mice and their 24 wild-type parents (C57BL6/N

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☐ Initial submission ☒ Revised version ☐ Final submission

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Experimental design

1. Sample size

Describe how sample size was determined.

> Sample size was largely constrained by resources, but was large enough to distinguish expected differences between positive and negative controls.

2. Data exclusions

Describe any data exclusions.

> For imaging experiments, only cells with healthy morphology were selected as targets. For data shown in Suppl. Figures SN2.1 and SN5.1-SN9.1, which represent the earliest collected data, 10 cells were imaged to establish a signal pattern. Cells that had dramatically outlying patterns (0 to 2 cells out of 10) were excluded as unhealthy.
> No recorded data were excluded from the analysis for Figure 1 and the other Suppl. Figures, except for 2 cells out of 32 in Fig. 1c which were unhealthy already at the beginning of the experiment.

3. Replication

Describe whether the experimental findings were reliably reproduced.

> No replication attempts failed.

4. Randomization

Describe how samples/organisms/participants were allocated into experimental groups.

> Cells were selected based on their presumed healthy morphology and size, cells undergoing various stages of apoptosis or obvious stress were avoided

5. Blinding

Describe whether the investigators were blinded to group allocation during data collection and/or analysis.

> No blinding was performed

Note: all studies involving animals and/or human research participants must disclose whether blinding and randomization were used.

6. Statistical parameters

For all figures and tables that use statistical methods, confirm that the following items are present in relevant figure legends (or in the Methods section if additional space is needed).

n/a	Confirmed	
☐	☒	The exact sample size (n) for each experimental group/condition, given as a discrete number and unit of measurement (animals, litters, cultures, etc.)
☐	☒	A description of how samples were collected, noting whether measurements were taken from distinct samples or whether the same sample was measured repeatedly
☐	☒	A statement indicating how many times each experiment was replicated
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☐	☒	A description of any assumptions or corrections, such as an adjustment for multiple comparisons
☐	☒	The test results (e.g. P values) given as exact values whenever possible and with confidence intervals noted
☐	☒	A clear description of statistics including central tendency (e.g. median, mean) and variation (e.g. standard deviation, interquartile range)
☐	☒	Clearly defined error bars

See the web collection on statistics for biologists for further resources and guidance.

Software

Policy information about availability of computer code

7. Software

Describe the software used to analyze the data in this study.	Initial processing was carried out in FIJI and Matlab; data were analyzed with Microsoft Excel and Prism; statistical analysis was performed in R.

For manuscripts utilizing custom algorithms or software that are central to the paper but not yet described in the published literature, software must be made available to editors and reviewers upon request. We strongly encourage code deposition in a community repository (e.g. GitHub). *Nature Methods* guidance for providing algorithms and software for publication provides further information on this topic.

Materials and reagents

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8. Materials availability

Indicate whether there are restrictions on availability of unique materials or if these materials are only available for distribution by a for-profit company.	The availability of the materials used is described in the manuscript, all materials that are not available commercially, are available upon request

9. Antibodies

Describe the antibodies used and how they were validated for use in the system under study (i.e. assay and species).	No antibodies were used in this study.

10. Eukaryotic cell lines

a. State the source of each eukaryotic cell line used.	ATCC HeLa CCL2 and Cos-7 CRL-1651
b. Describe the method of cell line authentication used.	The cells were freshly purchased from ATCC directly
c. Report whether the cell lines were tested for mycoplasma contamination.	We used Primocin (Invivogen) during cell growth which is active against mycoplasmas in the initial experiments
d. If any of the cell lines used are listed in the database of commonly misidentified cell lines maintained by ICLAC, provide a scientific rationale for their use.	No ICLAC listed cell line was used.

Animals and human research participants

Policy information about studies involving animals; when reporting animal research, follow the ARRIVE guidelines

11. Description of research animals

Provide details on animals and/or animal-derived materials used in the study.	N/A

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12. Description of human research participants

Describe the covariate-relevant population characteristics of the human research participants.

N/A