Optimizing imaging speed and excitation intensity for single-molecule localization microscopy

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High laser powers are common practice in single-molecule localization microscopy to speed up data acquisition. Here we systematically quantified how excitation intensity influences localization precision and labeling density, the two main factors determining data quality. We found a strong trade-off between imaging speed and quality and present optimized imaging protocols for high-throughput, multicolor and three-dimensional single-molecule localization microscopy with greatly improved resolution and effective labeling efficiency.

Single-molecule localization microscopy (SMLM) techniques such as (f)PALM and (d)STORM provide unmatched spatial resolution among fluorescence microscopy techniques1–5. As fluorophore blinking kinetics depend on the excitation intensity, the combination of high excitation laser intensity and fast detection can speed up data acquisition by orders of magnitude4–6. Powerful lasers are therefore common practice; although, until now, no clear consensus on the impact on SMLM image quality has been reached. The photon count per localization is the main determining factor of localization precision. Investigating a variety of labels, different groups reported no substantial influence of the excitation intensity8,9. The second important factor determining SMLM image quality is the labeling efficiency or density8, which has been estimated via photobleaching measurements9,10 or localization density9,11,12.

Here, we revisited the effects of excitation efficiency on image quality using a recently developed, highly quantitative assay based on nuclear pore complexes (NPCs) as reference standards13. We found that the two main factors determining SMLM image quality, that is localization precision and labeling efficiency8,9, strongly deteriorate with high excitation intensity and, thus, imaging speed. We systematically investigated the effect of imaging speed on image quality for a variety of fluorophores, labeling approaches and imaging buffers. We varied the excitation intensity over three orders of magnitude, while adjusting the exposure time inversely to keep a constant light dose per frame, resulting in optimal average on times of the fluorophores of a few frames. We merged localizations persistent over consecutive frames to measure the total photon count per localization, which we find is largely independent of the exposure time (Extended Data Fig. 1). In addition, the precise stoichiometry and eightfold symmetric arrangement of the reference protein Nup96 in the NPC allowed us to quantify absolute effective labeling efficiencies (fraction of target proteins that are detected during the image acquisition), as well as the number of localizations per fluorophore14 (Supplementary Table 1).

For the popular organic dye Alexa Fluor 647 (AF647, ref. 11) in a common oxygen depleting and thiol containing blinking buffer (dSTORM approach), fast imaging decreased the photon count per localization by a factor of ten (Fig. 1a), resulting in a threefold deteriorated localization precision and eightfold deteriorated Fourier ring correlation resolution14 (Fig. 1b). The effective labeling efficiency decreased by a factor of three (Fig. 1c), leading to sparse images (Fig. 1d), suggesting photobleaching during the initial off-switching phase, in which fluorophores are pushed into their dark state and cannot yet be localized due to high density of emitting fluorophores. Fluorophores that are irreversibly bleached during this phase are lost and reduce the effective labeling efficiency. In contrast, fluorophores bleached during the subsequent single-molecule imaging are likely to have emitted a sufficient number of photons to be localized and thus have little impact on the effective labeling efficiency.

To test this hypothesis, we performed the initial off-switching at different intensities (Fig. 1e and Supplementary Fig. 1), while keeping the conditions for subsequent single-molecule imaging identical. The effective labeling efficiency increased by up to a factor of two for low-intensity off-switching. Consequently, NPCs appeared more densely labeled and, thus, seem more complete in the reconstructed image (Fig. 1f). This observation has huge implications for the experimental design: In contrast to common practice12, the initial off-switching should be performed with low intensities to retain a high effective labeling efficiency, otherwise up to half of the fluorophores are bleached before single-molecule detection even begins.

Our findings can be qualitatively illustrated with simple models describing fluorophore switching in dSTORM16,17 in presence of bleaching (Extended Data Fig. 2 and Supplementary Note 2). The triplet state is long-lived in oxygen depleted buffers18, which leads to saturation of the fluorescence emission and thus decreased photon counts with high intensities. Photobleaching occurs predominantly from this triplet state18, leading to an intensity dependent bleaching that competes with transition into the dark state and recovery of the bright state. Thus, photobleaching becomes dominant for high intensities, reducing on times, photon counts and the number of localizations per fluorophore. Indeed, for low intensities, we could observe individual AF647 fluorophores five to ten times, while high intensities resulted in virtually no reactivations (Fig. 1c).

We tested the generality of our observations by investigating the effect of imaging speeds using different dSTORM imaging buffers (glucose oxidase/catalase (GLOX) buffer with cysteamine (MEA), b-mercaptoethanol (BME) or BME including refractive index matching), different tagging approaches (SNAP-tag, nanobody, antibody, endogenous fusion of fluorescent proteins), as well as different fluorophores (AF647, CF680, CF660C, Dy634, mMaple).

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We found that CF660C was much less prone to bleaching than any other dye, making it a favorable dye for long-term imaging. It enabled us to image whole cells via astigmatism-based three-dimensional dSTORM by stepping through multiple z positions in widefield illumination (Fig. 2a and Supplementary Videos 1 and 2). This is very challenging when using other dyes, as the acquisition of each image plane bleaches fluorophores in all planes (Extended Data Fig. 4) and has before been tackled by sophisticated excitation/detection schemes of different light-sheet geometries.21,22 With CF660C, on the other hand, we could image about 1 million frames, covering a volume up to about 40×40×6 μm³ fitting an entire mitotic cell (Extended Data Fig. 5 and Supplementary Video 3). CF660C can well be combined with CF680 and AF647 for ratiometric three-color SMLM at medium to high intensities (Extended Data Figs. 6–10). All conditions (Fig. 1g) qualitatively showed a similar trend of strongly decreased photon counts (Fig. 1h) and effective labeling efficiencies (Fig. 1i) for fast, high-intensity imaging.

Fig. 1 | Effect of laser intensity and imaging speed on image quality. a–c, Mean number of photons per localization (a), median localization precision and Fourier ring correlation (FRC) resolution (b) and effective labeling efficiency and mean number of localizations per fluorophore for AF647 in BME-based buffer as a function of the excitation intensity and single frame exposure time (c). d, SMLM images of Nup96-SNAP-AF647 corresponding to data shown in a–c. e, Effective labeling efficiency as a function of different off-switching intensities, recorded at identical imaging intensities of 61 kW cm⁻². f, SMLM images of Nup96-SNAP-AF647 corresponding to data shown in e. g, SMLM images of Nup96 using different buffers or labeling approaches recorded at 6.4 kW cm⁻² (organic dyes) or 1.9 kW cm⁻² (mMaple). h, i, Mean number of photons per localization (h) and effective labeling efficiency as a function of excitation intensity for different buffers or labeling approaches (i). All data taken on U2OS cells. Error bars indicate mean ± s.d. See Supplementary Table 1 for sample size and replicates. See Supplementary Figs. 2–10 for detailed data of individual conditions. Scale bars, 100 nm.
For low intensities, however, CF660C and CF680 exhibit a fraction of dim and long-lived states, rendering them unsuitable for SMLM (Extended Data Fig. 3 and Supplementary Video 5). On the other hand, both AF647 and Dy634 showed excellent performance at low-intensity conditions for highest-quality two-color SMLM (Fig. 2b and Extended Data Fig. 7).

Lowest intensities result in very high photon counts and numerous localizations per fluorophore, allowing us to approach the resolution of DNA-PAINT by dSTORM, albeit without suffering from high background inherent to DNA-PAINT and without the need for TIRF/HILO illumination. This allowed us to visualize the four individual Nup96 proteins per symmetric unit of the NPC (Fig. 2c,d and Supplementary Video 6) only 12 nm apart, a resolution that had been deemed out of reach for SMLM. Compared to recent three-dimensional (3D) DNA-PAINT measurements on the NPC, we could more than double the effective labeling efficiencies to 65% (Fig. 1c) with a comparable resolution.

Fig. 2 | Optimization of various imaging applications. a, Whole-cell 3D reconstruction of microtubules stained with CF660C from 900,000 frames recorded at 24 kW cm\(^{-2}\) excitation intensity and 83-Hz framerate. Top-view projection and orthogonal slices through the positions indicated by the dashed line. b, Two-color ratio metric 3D imaging of microtubules stained with AF647 (red) and clathrin stained with Dy634 (turquoise), recorded at 1.6 kW cm\(^{-2}\) excitation intensity and 8.6 Hz framerate. Top-view projection and orthogonal slices through the indicated positions. c, 3D imaging of Nup96-SNAP-AF647 recorded at 0.36 kW cm\(^{-2}\) and 2 Hz framerate. d, Magnified images of individual NPCs, as indicated in c, show individual Nup96 proteins per symmetric unit. e, Live-cell PALM of Nup96-mMaple recorded in 20 s at a 70-Hz framerate. Representative results are shown from \(n=3\) and \(n_c=5\) (a), \(n=2\), \(n_c=4\) (b), \(n=4\), \(n_c=4\) (c,d) and \(n=2\), \(n_c=4\) (e) experiments. \(n\) denotes the number of independent experiments and \(n_c\) denotes the number of imaged cells. All data taken on U2OS cells. Scale bars, 10 \(\mu\)m (a), 1 \(\mu\)m (b,c,e), 100 nm (b(i)-(iii), d).
Buffer acidification is known to limit long imaging times that are necessary when using low intensities by altering fluorophore properties. Tight chamber sealing can overcome this issue\(^2\). Simple parafilm sealing excluding any air bubbles enabled us to use the conventional GLOX buffer for more than 24 h without a substantial drop in pH or deteriorated data quality (Extended Data Fig. 8 and Supplementary Video 7).

Slow imaging is not an option for live-cell SMLM, where the biological process of interest dictates the imaging speed. As genetically encoded photoconvertible fluorescent proteins are labels of choice for live-cell imaging, we also tested the performance of mMaple for PALM at varying imaging speeds (Supplementary Fig. 10). Unfortunately, we found the same trade-off between imaging speed and image quality for mMaple as for organic dyes, in line with previous findings by Jones et al. for mEOS2, who attributed the loss in labels to bleaching from the unconverted state\(^6\). Still, an excitation intensity of \(\sim 7.5\) kW cm\(^{-2}\) and a framerate of \(\sim 70\) Hz represented a good compromise for mMaple, with high localization precision at only a moderate loss of effective labeling efficiency. This allowed us to resolve the circular structure of the NPC arrangement of Nup96 in living cells in only 20 s of acquisition time (Fig. 2e and Supplementary Video 8). We note that with these intensities phototoxicity cannot be excluded\(^8\).

In conclusion, we identified a clear trade-off between image quality and imaging speed in SMLM with slow imaging resulting in exceptional resolution and effective labeling efficiency. These findings were surprising in light of a recent report\(^1\), but a reanalysis of that data could resolve the contradiction and attribute it to suboptimal matching of exposure time and excitation intensity, which impeded correct merging of localizations (Extended Data Fig. 9).

If high throughput is required, our findings provide a guideline on how to choose the best compromise between imaging speed and quality. Specifically, we could identify CF660C as a dye with increased photo stability, which retains high effective labeling efficiencies under fast imaging conditions and allowed us to assemble whole-cell super-resolution data with optimal labeling densities in all slices. Optimizing the composition of the imaging buffer specifically at high excitation intensities has the potential to further improve image quality.

We found that the common practice of performing the initial switching-off phase with high laser powers bleaches up to half of the fluorophores before they can be localized even once. Thus, to retain high labeling efficiencies, the initial switch-off should be performed with low laser powers.

In light of the recent community effort to enhance SMLM via optically more and more complex detection schemes\(^2\), our results demonstrate a complementary and much simpler approach without optical modifications. Shifting to low laser powers will enable many laboratories to easily and directly improve their data quality.

### Online content
Any methods, additional references, Nature Research reporting summaries, source data, extended data, supplementary information, acknowledgements, peer review information; details of author contributions and competing interests; and statements of data and code availability are available at https://doi.org/10.1038/s41592-020-0918-5.

Received: 25 February 2020; Accepted: 13 July 2020; Published online: 17 August 2020

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**BRIEF COMMUNICATION**

**NATURE METHODS**

**VOL 17 | SEPTEMBER 2020 | 909–912 | www.nature.com/naturemethods**
Methods

Sample seeding. Before seeding of cells, high-precision 24-mm-round glass cover slips (no. 1.5H, catalog no. 117640, Marienfeld) were cleaned by placing them overnight in a methanolic/hydrochloric acid (50/50) mixture while stirring. After that, the cover slips were repeatedly rinsed with water until they reached a neutral pH. They were then place overnight into a laminar flow cell culture hood to dry them before finally irradiating the cover slips by ultraviolet light for 30 min.

Cells were seeded on clean glass cover slips 2 d before fixation to reach a confluency of about 50–70% on the day of fixation. They were grown in growth medium (DMEM, catalog no. 11880-02, Gibco) containing 1 mM MEM NEAA (catalog no. 11140-035, Gibco), 1× Glutamax (catalog no. 35050-038, Gibco) and 10% (v/v) fetal bovine serum (catalog no. 10270-106, Gibco) for approximately 2 d at 37 °C and 5% CO2. Before further processing, the growth medium was aspirated and samples were rinsed twice with PBS to remove dead cells and debris. Unless otherwise stated, all experimental replicates were performed on cells of a different passage with separated sample preparation.

Imaging buffers. The following buffers were used to image the indicated samples.

GLOX + MEA. GLOX buffer supplemented with MEA was used to image Nup96-SNAP-AF647. GLOX + MEA contained 50 mM of Tris/HCl pH 8, 10 mM of NaCl, 10% (v/w) d-glucose, 40 µg/ml of glucose oxidase, 40 mM of BME and glycerol for refractive index matching (n = 1.5). GLOX buffer supplemented with MEA was used to image Nup96-mEGFP-AB-AF647, Nup96-mEGFP-AB-CF660C, GLOX buffer supplemented with MEA was used to image Nup96-mMaple samples (live and fixed cells). GLOX buffer supplemented with MEA was used to image Nup96-mEGFP samples.

Imaging buffers. Preparations of Nup96-mMaple samples (live and fixed cells). Growth medium with HEPES contained 1% (w/v) glucose catalase and 143 mM of BME in H2O.

Preparation of Nup96-SNAP samples. Nup96-SNAP-tag cells (catalog no. 300444, CLS Cell Line Service) were fixed analogous to the procedure described for microtubule staining. Successive blocking was done for 30 min in 2% (w/v) BSA in PBS and 30 min in Image-iT FX Signal Enhancer before the sample was incubated in a mixture of rabbit antibodies against clathrin light chain (diluted 1:100, catalog no. sc-28276, Santa Cruz Biotechnology) and clathrin heavy chain (diluted 1:300, catalog no. ab21679, Abcam) in 1% w/v BSA, 300 mM Tris/HCl pH 8 in 1% (v/v) D2O. Washing of three times for 5 min each.

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DTT in PBS for 1 h to stain Nup96-SNAP-tag. Cells were washed three times for 5 min with PBS and subsequently blocked with 5% (v/v) normal goat serum (NGS) (catalog no. PCNSS006, lifeTech) in PBS for 1 h. Primary antibody labeling against the chromdomain-binding nucleoporin ELYS was achieved by incubation with anti-AHCTIF1 (HPA031658, Sigma Aldrich) diluted 1:30 in 5% (v/v) NGS in PBS for 1 h. Coverslips were washed three times times for 5 min with PBS to remove unbound antibody and subsequently stained with CF660C labeled antirabbit antibody (catalog no. 20183, Biotium) diluted 1:50 in PBS containing 5% (v/v) NGS for 1 h. After three washes with PBS for 5 min, the sample was postfixed for 30 min using 2.4% (w/v) FA in PBS, rinsed with PBS, quenched in 50 mM of NH$_4$Cl for 5 min and rinsed three times for 5 min with PBS. Shortly before imaging, the sample was incubated for 10 min with 1:5,000 diluted WGA-CF680 (catalog no. 29029-1, Biotium) in PBS, rinsed three times with PBS and mounted onto a custom-manufactured sample holder in appropriate imaging buffer (see Imaging buffers). The holder was sealed with parafilm.

**Microscope setup.** The custom-build widefield microscope used for acquiring all single-color data is described in Supplementary Note 1 and shown in Supplementary Fig. 12.

**SMLM data acquisition for intensity titration.** For intensity dependent measurements, we recorded a region of 128 by 128 pixels in the center of the camera chip. We used the global exposure trigger to illuminate the sample only during the time in which all lines of the rolling shutter sCMOS camera were exposed simultaneously. This effective laser exposure time was shorter than the camera exposure time set in Micro-Manager. We chose the excitation intensities such that the product of the effective laser exposure time and intensity stayed constant for all conditions of individual fluorophores (Supplementary Table 1 and Supplementary Dataset 1). For the fastest acquisition settings, we kept the camera exposure time setting on 3 ms each but triggered the acquisition every 2–9 ms using an EM-CCD camera in frame transfer mode, mean laser intensity 2.5 times the camera exposure time set in Micro-Manager. For some settings, we kept the camera exposure time setting on 3 ms each but triggered the acquisition every 2–9 ms using an EM-CCD camera in frame transfer mode, mean laser intensity 2.5 times the camera exposure time set in Micro-Manager.

**SMLM data acquisition for off-switching intensity titration.** Different off-switching conditions were realized by recording, but not analyzing, 100 frames each at the respective pair of off-switching intensity and camera exposure time (Supplementary Table 1). Hence, the effective laser off-switching times were slightly lower, but the duration of the off-switching phase was limited by the camera exposure time. We then changed both the intensity, camera exposure time and effective laser exposure time to either 24 kW cm$^{-2}$, 9 and 7.45 ms or 61 kW cm$^{-2}$, 3 and 1.25 ms, respectively. Note that the latter condition (shown in Fig. 1e,f) does not fit our otherwise applied standard of keeping the product of effective laser exposure time and intensity constant, but was instead chosen to effectively fit the image acquisition conditions of a recent study by Barentine et al.

**SMLM data acquisition for other experiments.** Whole-cell 3D images were recorded at a set camera exposure time of 12 ms, corresponding to an effective single-frame laser exposure time of about 7.6 ms. A mean excitation laser intensity of 24.3 kW cm$^{-2}$ and UV activation intensity of 0.011 kW cm$^{-2}$ were used. Long-term imaging for comparison of AF647 and CF660C was recorded with an average point-spread function (PSF) model calculated from tens of stacks using previously published algorithms with custom software written in MATLAB (super-resolution microscopy analysis platform, SMAP$^3$), available as open source at github.com/ries/SMAP.

**Fitting and postprocessing.** Two-dimensional data were fitted with a symmetric Gaussian PSF model with the FSF size, x, y, photons per localization and the background as free-fitting parameters using maximum likelihood estimation$^{30}$. 3D data were fitted using an experimentally derived PSF model from the 3D bead calibration with x, y, z, photons per localization and the background as free-fitting parameters using maximum likelihood estimation$^{30}$. The data were then corrected for drift in x, y and z by a custom algorithm based on redundant cross-correlation. From the spread of the redundant displacements we estimated the accuracy of the drift correction to be better than 1.5 nm in x and y and 2 nm in z. Before analysis, the localizations were filtered based on localization precision (0–10 nm for dSTORM data of organic dyes shown in Fig. 1, 0–15 nm for PALM data of mSnap-labeled Nup96 in Fig. 1) to exclude extraordinarily dim localizations, PSF size (laterally 0–160 nm for dSTORM data of organic dyes or laterally 0–160 nm for PALM data of mMaple, for two-dimensional data only) to remove out of focus localizations, frames (from 1,000 until completion, except for antibody- and nanobody-staining data used for power titration measurements where the acquisition was stopped after 31,000 frames; no frame filter was applied to mMaple data shown in Fig. 1) and photobleaching/decay and position to reject molecules far away from the focal plane.

**Long-term buffer pH stability.** We measured the pH value directly before sealing the chamber, and directly after opening the chamber 24 h later. For comparison, 1 ml of freshly mixed GLOBE and EM buffer was stored for 24 h in an area of 0.8 cm$^2$ for 24 h. Storage was performed at room temperature identical to all image acquisitions. Indicator paper was used to determine the pH value (MColorPheat for pH6.5–10.0, Merck, or pH0–14 Universal indicator, Merck).

**3D bead calibration.** Tetra-Speck beads (0.7 μm in stock, catalog no. 77279, Thermofisher) were diluted in 360 μl of H$_2$O, mixed with 40 μl 1 M MgCl$_2$, and put on a coverslip in a custom-manufactured sample holder. After 10 min, the mix was replaced with fresh 0.6 μl H$_2$O. For experiments where the Si-objective was used, the mix was replaced by 400 μl refractive index matched buffer consisting of 55% glycerol (85%) and 45% H$_2$O. Using Micro-Manager, about 20 positions on the coverslip were defined and the PSF size was measured using an EM-CCD camera in frame transfer mode, a mean excitation laser intensity of 1.6 kW cm$^{-2}$, but at variable pulse-widths.

**Crosstalk quantification.** To separate the colors in ratiometric imaging, the localizations were filtered and the ratio between photons from both channels was plotted. The channels for each fluorophore were assigned manually by drawing ROIs in the plot. This assignment was saved and used later for crosstalk determination.
and the localizations identified as AF647, Dy634 and not assigned (that is, rejected) were counted.

For three-color imaging, the crosstalk was identically determined, but based on the individually stained structures as described above. Nup96-SNAP via BG-AF647 staining, ELYS via indirect immunostaining with CF660C and WGA-CF680).

**Segmentation of nuclear pores.** All images used in this work that are based on Nup96-derived data, were segmented automatically in SMAP. For this, reconstructed images were convolved with a kernel consisting of a ring with a radius corresponding to the radius of the NPC, convolved with a Gaussian. Local maxima over a user-defined threshold were treated as possible candidates. Candidates were cleaned up by two additional steps. (1) We fitted the localizations corresponding to each candidate with a circle and excluded structures with a very small (<50 nm for antibody-labeled data, <40 nm for other) or very large (>80 nm for antibody-labeled data, >70 nm for other) ring radius. (2) Localizations were refitted with a circle of fixed radius to determine the center coordinates. Structures were rejected if more than 25% of localizations were closer than 40 nm to the center (50 nm for antibody-labeled data) or if more than 40% of localizations were further away than 70 nm (80 nm for antibody-labeled data) from the center, as these typically did not visually resemble NPCs or were two adjacent wrongly segmented NPCs.

**Determination of effective labeling efficiencies.** Calculation of effective labeling efficiencies was done on all successfully segmented NPCs and followed an automated six-step protocol. (1) Localizations were fitted with a ring with a fixed radius corresponding to the mean radius determined before. Additionally, localization coordinates were converted into polar coordinates $\varphi$, $r$. (2) Localizations closer to the center than 30 nm (50 nm for antibody-labeled data) or further away than 70 nm (80 nm for antibody-labeled data) were excluded as background localizations. (3) Rotational alignment of structures was achieved by minimizing $\varphi_{\text{rot}} = \arg\min_{\varphi} \left( \varphi_{\text{rot}} - \varphi \right)$ mod $\pi$. (4) The structures were overlayed with a mask consisting of eightfold symmetric segments, recapitulating the arrangement of Nup96 in the NPC. We counted the number of segments containing a localization from a histogram of $\varphi$, with a bin width of $\frac{\pi}{8}$ and a start bin of $\frac{\pi}{8}$. (5) We then constructed a histogram of the number of detected nonempty segments in all NPCs in the dataset and fitted it using a probabilistic model as described before with the effective labeling efficiency as a free-fitting parameter. (6) Statistical error was determined by bootstrapping with 20 resampled data sets.

**Determination of the number of localizations per fluorophore.** The number of localizations per fluorophore was calculated by dividing the number of localizations per NPC by the effective labeling efficiency times 32; that is, the number of Nup96 molecules per NPC.

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

**Data availability**
All processed data (lists of localizations) and for each condition at least one example file of raw data (camera frames of blinking fluorophores) are deposited on BioStudies (https://www.ebi.ac.uk/biostudies/S-BSST425) under accession number S-BSST425. Source data are provided with this paper.

**Code availability**
The software for the data acquisition and analysis used in this paper is available at github.com/jdeschamps/htSMLM and github.com/jries/SMAP, respectively.

**References**
30. Li, Y. et al. Real-time 3D single-molecule localization using experimental point spread functions. Nat. Methods 15, 367–369 (2018).

**Acknowledgements**
We thank A. Rahmani and V. Dunsing for assistance in the data acquisition and processing. We thank Y. Lin and J. Bewersdorf for sharing their raw data and discussing the reanalysis. This work was supported by the European Research Council (grant no. ERC CoG-724489 to J.R.), the National Institutes of Health Common Fund 4D Nucleome Program (grant no. U01 EB021223 to J.R.), the Human Frontier Science Program (grant no. RGY0065/2017 to J.R.), the Engelhorn Foundation (Postdoctoral Fellowship to R.D.) and the European Molecular Biology Laboratory.

**Author contributions**
R.D., M.K. and J.R. conceived the project. R.D. built the main optical setup. M.K., A.S. and U.M. prepared the samples. R.D., M.K. and A.S. acquired and analyzed the data. J.D. wrote the acquisition software. J.R. wrote the analysis software and performed the simulations. R.D., M.K., A.S. and J.R. wrote the manuscript with input from all authors.

**Competing interests**
The authors declare no competing interests.

**Additional information**
Extended data is available for this paper at https://doi.org/10.1038/s41592-020-0918-5.

Supplementary information is available for this paper at https://doi.org/10.1038/s41592-020-0918-5.

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Peer review information Rita Strack was the primary editor on this article and managed its editorial process and peer review in collaboration with the rest of the editorial team.

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Extended Data Fig. 1 | Effect of exposure time for constant excitation intensity in simulations. Realistic simulations of camera frames (see Supplementary Note 2) for a constant excitation intensity when varying the exposure time over four orders of magnitude. Our standard analysis workflow was used to merge localizations in consecutive frames, stemming from the same fluorophore, and to determine the photons per localization, localization precision, on-time and background per localization per pixel. These simulations show an optimal exposure time that is shorter than the mean on-time, which leads to the highest localization precision. Around this value, the photons per localization and the localization precision display only minimal dependence on the exposure time. For much shorter exposure times, however, merging fails due to missed localizations and large localization errors, reducing photons per localization and deteriorating the localization precision. For exposure times longer than the mean on-time, the background increases linearly with the exposure time, whereas the photons per localization stay constant, also leading to a deteriorated localization precision. Simulation parameters: Mean on-time 30 ms, mean photon emission rate 66 667 Hz, camera read-noise 1.8 e⁻.
Extended Data Fig. 2 | Model for blinking and bleaching of dSTORM dyes. Model 1 (red curves, inspired by van der Linde et al, Photochem Photobiol Sci, 2011 and Lin et al, PLOS ONE 2015) assumes off-switching from the triplet state, whereas Model 2 (blue curves, inspired by Dempsey et al, Nature Methods, 2011) assumes intensity dependent off-switching from the bright state. B: bright state, T: triplet state, D: dark state, X: bleached state. The graphs show Monte Carlo (MC) simulations (solid line) and numerical integration of partial differential equations (PDE, dashed lines) in comparison to data (dots) for the number of photons per localization, the effective labeling efficiency, the localizations per fluorophore and the on-time. The simulations are described in detail in Supplementary Note 2. For MC simulations, we simulated 100,000 fluorophores for each intensity value, we calculated photons per frame and merged and filtered fluorophores similar to our experimental data analysis. Rate constants from literature Lin et al, PLOS ONE, 2015: \( k_{bt} = 10 \text{cm}^2 \text{s}^{-1} \text{W}^{-1} \), \( k_{tb} = 70 \text{s}^{-1} \), \( k_{td} = 15 \text{s}^{-1} \). Rate constants that were chosen to result in similarity of simulations with the experiment: \( k_{db} = 2 \text{cm}^2 \text{s}^{-1} \text{W}^{-1} \), \( k_{bd} = 0.1 \text{cm}^2 \text{s}^{-1} \text{W}^{-1} \), \( k_{dx} = 0.01 \text{cm}^2 \text{s}^{-1} \text{W}^{-1} \), \( k_{dx} = 0.0002 \text{cm}^2 \text{s}^{-1} \text{W}^{-1} \). Exposure time \( t_{exp} = 0.176 \text{s} \text{W/cm}^2 \), off-switching time \( t_{offswitch} = 30 \text{s} \text{W/cm}^2 \) with excitation intensity \( I \).
Extended Data Fig. 3 | Raw data. Four consecutive raw camera frames for the different dyes used in this manuscript, comparing different exposure times and excitation intensities. See Supplementary Table 1 for sample size and replicates for the individual conditions. Size of each image 100 x 100 pixels, scaling 0-900 photons (dyes), 0-300 photons (mMaple). Scale bars 1 µm.
Extended Data Fig. 4 | See next page for caption.
Extended Data Fig. 4 | Whole cell imaging with AF647 and CF660C. Whole cell 3D images of microtubules in U2OS cells immunostained with AF647 (left) or CF660C (right). Individual slices at increasing, subsequently recorded z-positions are shown. The z-distance between individual slices was 300 nm and 50,000 frames were recorded for each slice. The image reconstruction shows a representative result of \( n_c = 2 \) individual cells from the same preparation (N = 1). Excitation intensity 24 kW/cm², effective exposure time 7.5 ms.
Extended Data Fig. 5 | 3D imaging of a mitotic cell with CF660C. Volume 3D imaging of microtubules stained with CF660C recorded at 24 kW/cm² excitation intensity and 7.5 ms effective exposure time covers a volume of about 40 µm × 40 µm × 6 µm and, hence, enables SMLM imaging of whole mitotic cells. Top-view projection and orthogonal projections over the indicated regions. See Supplementary Video 3 for a 3D reconstruction. The image reconstruction shows a representative result of n_c = 2 individual cells from two different preparations (N = 2).
Extended Data Fig. 6 | Three-color ratiometric 3D imaging. Three-color ratiometric 3D imaging of the nuclear pore complex. Nup96-SNAP is tagged with BG-AF647, ELYS is visualized by indirect immunostaining with CF660C and the central channel is stained with WGA-CF680. a, Lateral view of the lower nuclear envelope. b, Magnified region as indicated in a. c, Side view on region indicated in b. See Supplementary Video 4 for a 3D reconstruction corresponding to c. d, Crosstalk between different channels. Bars show the fraction of the true label being assigned to each channel. The image reconstruction shows a representative result of \( n_r = 3 \) individual cells from the same preparation (\( N = 1 \)). The crosstalk was determined from \( n_c = 6 \) individual measurements from the same preparation (\( N = 1 \)).
Extended Data Fig. 7 | Crosstalk in two-color ratiometric 3D imaging. Crosstalk in slow ratiometric imaging using Dy634 and AF647. Bars show the fraction of the true label being assigned to each channel. The crosstalk was determined from $n_c = 6$ individual measurements from the same preparation ($N = 1$).
Extended Data Fig. 8 | See next page for caption.
Extended Data Fig. 8 | GLOX+BME buffer long-term stability. Characterization of long-term stability of GLOX+BME imaging buffer. a, pH value of glucose oxidase/catalase buffer supplemented with 143 mM BME either in direct contact to ambient air or stored in a sealed chamber, measured directly after preparation and after 24 h. Results from six independent experiments each. Error bars denote the accuracy of the pH indicator strips. b, Effective labeling efficiency, c, photons per localization, and, d, localizations per fluorophore for $n_c = 4$ (before 24 h) and $n_c = 5$ (after 24 h) individual cells from the same preparation ($N = 1$) using a sealed chamber. The sample was stored on the microscope at room temperature. Error bars in b-d denote mean ± standard deviation. e, Representative image of Nup96-SNAP BG-AF647 before 24 h and, f, after 24 h storage in the imaging buffer. dSTORM data was recorded at 6.4 kW/cm². See Supplementary Video 7 for a time-lapse of the buffer response in an open and sealed chamber side by side. The image reconstructions show a representative result of $n_c = 6$ individual cells from one preparation ($N = 1$).
Extended Data Fig. 9 | Discrepancy to previous work by Lin et al. Lin et al. reported a nonmonotonic dependence of the photons per localization as a function of the excitation intensity (blue curve, original data read from the publication) for dSTORM imaging of immunostained microtubules. To investigate the discrepancy with our data (gray curve), that is monotonically decreasing photons per localization as a function of the excitation intensity, we partially \((n_t = 1\) for each intensity, \(N = 1\)) refitted and reanalyzed the original data kindly provided by Yu Lin and Joerg Bewersdorf. Using our fitter, our localization filter settings and our analysis pipeline, we find higher photons per localization but the same general trend (magenta curve). However, Lin et al. did not keep the product of excitation intensity and single frame exposure time constant. For our data it is constant with \(t_{ex} I = 0.18\) kW/cm\(^2\) s. Instead, Lin et al. used exposure times as short as 20 ms even for the lowest intensities for example resulting in \(t_{ex} I = 0.02\) kW/cm\(^2\) s. As a consequence, blink events easily persist over tens of frames, which makes grouping of localizations error prone. To virtually adjust the product of excitation intensity and exposure time for the data of Lin et al., we summed up single frames of the raw data before fitting (9 frames each for 1 kW/cm\(^2\), 5 frames each for 1.9 kW/cm\(^2\), 2 frames each for 3.9 kW/cm\(^2\)) or selected the conditions closest to our value chosen for the product (7.8 kW/cm\(^2\) to 97 kW/cm\(^2\)). Additionally, Lin et al. used a less effective setup of emission filters which we estimate to transmit 79 % less fluorescence than our excitation filters. Combining both the refit data of the virtually restored product of excitation intensity and exposure time as well as scaling up the photons by 79 % (green curve) is well in agreement with our results. For each intensity in our data, the mean value \(\pm\) standard deviation is shown over the data points representing individual cells. For our data, see Supplementary Table 1 for sample size and replicates.
Extended Data Fig. 10 | Effect of filtering on photon counts. The localization filters applied for organic dyes in this study influence the localization statistics. To visualize the influence, we applied different filter settings to the localizations and re-analyzed the data with respect to the photon counts. Though the absolute numbers change, the general trend of decreasing photon counts with increasing excitation intensity is maintained. These graphs are for the sample Nup96-SNAP-AF647 in 143 mM BME (Supplementary Fig. 2). See Supplementary Table 1 for sample size and replicates. Precision: localization precision filter, PSF: filter on fitted size of the PSF, llrel: filter on the normalized log-likelihood ratio. Error bars denote mean ± standard deviation.
Report on data availability

All manuscripts must include a data availability statement. This statement should provide the following information, where applicable:
- Accession codes, unique identifiers, or web links for publicly available datasets
- A list of figures that have associated raw data
- A description of any restrictions on data availability

All processed data (lists of localizations) and for each condition at least one example file of raw data [camera frames of blinking fluorophores] are deposited on BioStudies (https://www.ebi.ac.uk/biostudies/) under accession number S-BS51425.
Field-specific reporting

Please select the one below that is the best fit for your research. If you are not sure, read the appropriate sections before making your selection.

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For a reference copy of the document with all sections, see nature.com/documents/nr-reporting-summary-list.pdf

Life sciences study design

All studies must disclose on these points even when the disclosure is negative.

Sample size

For experiments involving NPCs, we did not do a sample size calculation. A minimum of 300 individual NPCs were analysed for each condition, which resulted in a low statistical variation. From previous simulations (https://doi.org/10.1038/s41592-019-0574-9), we determined that the above mentioned sample size was sufficient. Additionally, these NPCs were pooled from different cells on different coverslips, and for most conditions from multiple biologically independent measurements, i.e. different cell culture passage numbers and preparations.

Data exclusions

Only data from experiments that obviously failed (wrong microscope setup/out-of-focus/sample staining/wrong emitter activation/failed drift correction/failed 3D registration) were excluded. Exclusion criteria were predetermined.

Replication

All replications were successful. Detailed information about number of biologically independent replicates can be found in the corresponding figure legends and/or Supplementary Table 1.

Randomization

Samples were not randomized. Our experimental workflow did not allow randomization as imaging conditions and the analysis pipeline had to be adjusted according to the underlying sample (Methods).

Blinding

Blinding was not done. Our experimental workflow did not allow blinding as imaging conditions and the analysis pipeline had to be adjusted according to the underlying sample (Methods).

Reporting for specific materials, systems and methods

We require information from authors about some types of materials, experimental systems and methods used in many studies. Here, indicate whether each material, system or method listed is relevant to your study, if you are not sure if a list item applies to your research, read the appropriate section before selecting a response.

| Materials & experimental systems | Methods |
|---------------------------------|---------|
| n/a                             | n/a     |
| "X" Antibodies                  | "X" ChiP-seq |
| "X" Eukaryotic cell lines       | "X" Flow cytometry |
| "X" Palaeontology               | "X" MRI-based neuroimaging |
| "X" Animals and other organisms |         |
| "X" Human research participants |         |
| "X" Clinical data               |         |

Antibodies

Antibodies used

- Anti-GFP FluorTag-Q; mEGFP NanoTag Biotechnologies; #N0301, Clones 1H1, (1:50 dilution)
- Anti-GFP-IgG (rabbit) mEGFP MBL International, #598, Polyclonal, (1:250 dilution)
- Inhouse coupled anti-rabbit-AF647 (donkey) IgG, #711-005-152, Dianova, Polyclonal, (1:300 dilution)
- Inhouse coupled anti-rabbit-Dy634 (donkey) IgG, #711-005-152, Dianova, Polyclonal, (1:300 dilution)
- Anti-rabbit-CF660C IgG, Biotium, #20183, Polyclonal, (1:300 dilution)
- Anti-alpha-Tubulin IgG (mouse), Sigma Aldrich, #T6074, Polyclonal, (1:500 dilution)
- Anti-mouse-AF647 IgG, Thermo Fisher, #21236, Polyclonal, (1:500 dilution)
- Anti-mouse-CF660C IgG, Biotium, #20050-1, Polyclonal, (1:500 dilution)
- Anti-clathrin heavy chain (rabbit) IgG, abcam, #ab21679, Polyclonal, (1:300 dilution)
- Anti-clathrin light chain (rabbit) IgG, Santa Cruz Biotech, #sc-28276, Polyclonal, (1:100 dilution)
- Anti-AHCT1 (rabbit) IgG, Sigma Aldrich, #HPA031658, Polyclonal, (1:30 dilution)

Validation

- Anti-GFP FluorTag-Q; mEGFP NanoTag Biotechnologies; #N0301, Clones 1H1
- Wagner et al. (2019) Myosin VI Drives Clathrin-Mediated AMPA Receptor Endocytosis to Facilitate Cerebellar Long-Term Depression DOI:https://doi.org/10.1016/j.cerebres.2019.06.005
- Anti-GFP-IgG (rabbit) mEGFP MBL International, #598
- Engedal N, et al., All-trans retinoic acid stimulates IL-2-mediated proliferation of human T lymphocytes: early induction of cycl
Eukaryotic cell lines

Policy information about cell lines

Cell line source(s) All cell lines are available from CLS Cell Line Service, Eppelheim, Germany. U2OS Nup96 mMaple (catalog no. 300461) U2OS Nup96-SNAP-tag (catalog no. 300444) U2OS Nup96-mEGFP (catalog no. 300174)

Authentication Cell lines were not further authenticated.

Mycoplasma contamination Cell lines have been tested and are negative for mycoplasma contamination.

Commonly misidentified lines No commonly misidentified cell lines were used