Enhanced labeling density and whole-cell 3D dSTORM imaging by repetitive labeling of target proteins

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With continuing advances in the resolving power of super-resolution microscopy, the inefficient labeling of proteins with suitable fluorophores becomes a limiting factor. For example, the low labeling density achieved with antibodies or small molecule tags limits attempts to reveal local protein nano-architecture of cellular compartments. On the other hand, high laser intensities cause photobleaching within and nearby an imaged region, thereby further reducing labeling density and impairing multi-plane whole-cell 3D super-resolution imaging. Here, we show that both labeling density and photobleaching can be addressed by repetitive application of trisNTA-fluorophore conjugates reversibly binding to a histidine-tagged protein by a novel approach called single-epitope repetitive imaging (SERI). For single-plane super-resolution microscopy, we demonstrate that, after multiple rounds of labeling and imaging, the signal density is increased. Using the same approach of repetitive imaging, washing and re-labeling, we demonstrate whole-cell 3D super-resolution imaging compensated for photobleaching above or below the imaging plane. This proof-of-principle study demonstrates that repetitive labeling of histidine-tagged proteins provides a versatile solution to break the ‘labeling barrier’ and to bypass photobleaching in multi-plane, whole-cell 3D experiments.

Optical imaging beyond the diffraction limit has become part of the standard repertoire in cell biology. Super-resolution microscopy is suitable to visualize the spatial arrangement of proteins with near-molecular resolution and to quantify the composition of protein complexes. Crucial for all advanced super-resolution microscopy experiments are robust and ideally stoichiometric methods to label protein targets with fluorescent reporters that achieve a high labeling efficiency. While this seems to be given when fusing the protein of interest with a fluorescent protein, either in a knockout background or even better as a knock-in, the resolution is limited by the smaller number of photons typically emitted by fluorescent proteins until they photobleach. Furthermore, not every fluorescent fusion protein may mature into a photon-emitting state, potentially yielding labeled fusion proteins invisible in the experiment. The most commonly applied alternative strategy is labeling with small organic fluorophores, either by immunofluorescence, by introducing chemical tags, or by fluorophore-labeled ligands such as toxins that bind to a specific target. While the photon-yield of organic fluorophores is larger than that of fluorescent proteins and hence the achievable localization precision is smaller, some labeling strategies suffer from low efficiencies and unspecific binding, hampering the structural resolution. Average labeling densities of fluorophore-conjugated antibodies are in the range of 10–20%. Enzyme tags, such as the SNAP-tag, can reach a labeling efficiency of around 50%. An important reason for this observation may arise from the size of labeling probes such as antibodies limiting access to their binding sites, especially in regions of dense protein arrangements. Small probes can bypass this size limitation, e.g. the “small labeling pair” (SLAP) tag that builds on the specific interaction between a short peptide sequence, His6 or His10 and the trivalent tris-N-nitrilotriacetic acid (NTA) tag.

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plexed imaging with the same fluorophore. Another recent addition is FRET-PAINT16, which efficiently reduces photobleaching of fluorophores by replenishing fresh labels in each round of imaging. This approach is in particular useful for volume imaging with increased signal density. This approach is in particular useful for volume imaging with increased signal density. To demonstrate super-resolution of these images, we preferred localization of lamins underneath the nuclear membrane (near continuous line with infoldings) and at distinct nucleoplasmic sites (dots within the nucleus). Following imaging, we eluted the fluorophore probe from the sample using an imidazole-containing washing buffer (see Methods for a detailed description), re-labeled the sample with fresh NTA-AlexaFluor647, and recorded another dSTORM image (Fig. 2a–c). The repeated cycles of labeling, dSTORM imaging and elution, we demonstrate super-resolution microscopy with increased signal density. This approach is in particular useful for volume imaging as it re-compensates photobleaching of fluorophores by replenishing fresh labels in each round of imaging.

Results

We designed an experimental protocol consisting of multiple sequences of labeling, imaging, and elution of trisNTA-fluorophores targeting a His10-tagged protein (Fig. 1a). The nanomolar (nM) affinity interaction of the His10-tag with trisNTA allows for specific binding and an off-rate sufficiently slow for dSTORM imaging. Yet, washing the sample with a buffer containing 150 mM imidazole reliably removes the fluorophore label from the target without harming cellular structures. Hence, many cycles of labeling, imaging, elution, and re-labeling can be achieved. We implemented this configuration on an inverse microscope with two circulating pumps for semi-automatic fluid exchange (Fig. 1b).

To demonstrate the SERI concept, we expressed His10-mEGFP-LaminA in U2OS cells, stained the cells with trisNTA-AlexaFluor647 and recorded dSTORM images of single cells. The images shown in Fig. 2 reflect the preferred localization of lamins underneath the nuclear membrane (near continuous line with infoldings) and at distinct nucleoplasmic sites (dots within the nucleus). Following imaging, we eluted the fluorophore probe from the sample using an imidazole-containing washing buffer (see Methods for a detailed description), re-labeled the sample with trisNTA-AlexaFluor647, and recorded another dSTORM image (Fig. 2a–c). The repeated cycles of labeling, imaging, and elution generated multiple super-resolution images that can be merged into one image thereby achieving an increased signal density (Fig. 2d). To demonstrate super-resolution of these images, we determined the localization precision from each single staining round using the nearest-neighbor method19. We found an average localization precision of 24.1 ± 4.6 nm (mean ± SD), consistent with super-resolved images (a higher magnification image is not shown here because the lamina network is too dense to be resolved with this resolution, see19).
While this increase in signal density may reflect a higher degree of labeling by trisNTA-AlexaFluor647 binding to new target molecules in each round of labeling, further experiments are required to support such a mechanism. At this stage of a proof of concept, we point out at a clear increase in image contrast that is observed with SERI and is practically useful (compare Fig. 2a–c to Fig. 2d).

We next quantified the reliability of SERI by determining the number of single-molecule localizations per area in each round of imaging. We recorded dSTORM images of a U2OS cell expressing His_{10}-mEGFP-LaminA labeled with trisNTA-AlexaFluor647 before and after elution with imidazole, followed by illumination with high laser intensities to induce photo-bleaching of the remaining fluorophores (Fig. 2e). Elution with imidazole reduced the number of localizations to below 4% and additional photo-bleaching to even less than 1% of the original number of localizations in the labeled sample (Fig. 2f). The fraction of unbleached fluorophores after recording of the first 11,000 frames was estimated by using a bleaching model (see materials and methods). The number of single-molecule localizations for consecutive rounds of dSTORM imaging were similar for each round, yet accumulated with each round to yield a much higher total number of localizations (Fig. 2g).

For all re-staining experiments, we implemented a simple approach for liquid handling on our microscopy setup, thereby minimizing efforts of aligning image frames due to displacements of the sample. The stage coordinates were saved for each position and up to ten different positions per staining cycle were imaged. All cells could be re-identified and registered over different staining cycles. Each single round of imaging produced super-resolved images of LaminA that are well comparable to previously published super-resolved images of this structure using dSTORM imaging and the same small labeling pair. Additional rounds of staining were performed in a time-efficient way with elution for 5 min and re-staining with trisNTA-AlexaFluor647 for one hour. We provide proof of principle that SERI increases the signal density and thus image contrast when adding up localizations of all imaging rounds. After three rounds of labeling, imaging and elution, the cumulated signal intensity is approximately three times higher than in single experiments and has not yet reached saturation (Fig. 2f,g). In addition, we determined the signal-to-noise ratio of single super-resolution images and compared it to the cumulated image. We found that restaining and reimaging of the samples significantly increase the signal-to-noise ratio (see Methods, Supplementary Figure 1).

Another challenge in super-resolution microscopy is large volume imaging, which often suffers from photo-bleaching of fluorophores above or below the actual imaging plane. This challenge can be addressed with fluorescent probes that specifically target cellular structures as illustrated by imaging multi-cellular samples.
with fluorogenic PAINT-probes. Here, we explored how the SERI concept can provide a labeling density sufficient for 3D super-resolution imaging of volumes as large as whole-cells. We chose U2OS cells expressing His_{10}-mEGFP-LaminA to demonstrate whole-cell imaging, since the protein LaminA covers the whole nucleus and represents a large volume structure within these cells. After trisNTA-AlexaFluor647 staining, entire nuclei of U2OS cells expressing His_{10}-mEGFP-LaminA were imaged in 11 mutually overlapping slabs of approximately 900 nm thickness using astigmatism-based 3D dSTORM (Fig. 3a). This was achieved by using a piezo-based objective positioner calibrated to move the objective by approximately 550 nm during each imaging step. Thereafter, trisNTA-AlexaFluor647 was eluted with imidazole buffer and three more rounds of staining, imaging of 11 consecutive slabs in reverse order and elution were performed (Fig. 3b–c). Similar to single-plane imaging shown above, the labeling density increased continuously over several imaging rounds as evident in the cumulated image (Fig. 3f). The images show that laminA forms a continuous layer of about 200 nm thickness underneath the nuclear membrane (Fig. 3g,h). The pronounced bleaching of fluorophores situated, depending on the direction of stack acquisition, above or below the imaging plane, is evident in the z-intensity profiles of the individual measurements (Fig. 3i). Merging the individual imaging rounds strongly improved the labeling density in the z dimension (Fig. 3i). In conclusion, photobleaching of neighboring imaging slabs was compensated for by repetitive replenishment of the trisNTA-AlexaFluor647, thereby demonstrating the feasibility of large volume 3D super-resolution imaging at the scale of entire cells.

**Discussion**

We introduce a novel combined repetitive labeling and imaging approach with a small labeling pair of trisNTA-AlexaFluor647 and oligohistidine-tagged proteins. Our approach allows reversible targeting of proteins similar to DNA-PAINT, but does not require the use of antibodies. Antibody labeling suffers from a low labeling efficiency and a large distance between label and respective epitope of approximately 7.5 nm. In comparison, the distance between oligohistidine-tags and trisNTA-AlexaFluor647 is in the range of 1 nm, which places the fluorophore very close to the target protein. This is especially favorable as the localization precision constantly improves to the point of near-molecular resolution.

We chose His_{10}-mEGFP-LaminA for proof-of-principle experiments, because it easily allows to differentiate between background labeling (mostly within the nucleus) and labeling of the true structure that encompasses the nuclear envelope. In addition, LaminA staining allows imaging a large volume structure in U2OS cells, which demonstrates the capability of our method. We quantified the efficiency of SERI in multiple rounds of elution, bleaching, and re-staining: (i) the elution protocol reliably elutes more than 95% of the original labels; (ii) elution does not disrupt the underlying structure, because each imaging cycle could be reliably superimposed; (iii) labeling density increased with each round of labeling and imaging, yielding a more detailed image (Fig. 2g); (iv) SERI enables whole-cell super-resolution microscopy.

In conclusion, any other cellular protein could be imaged with the SERI approach and also other suitable labeling pairs could be used. It may depend on individual features of the protein such as abundance and spatial arrangement that will define successful application of SERI. Furthermore, small organic labels may produce signal background in SMLM experiments because of unspecific binding of the trisNTA-fluorophore conjugates. Experimental protocols that reduce unspecific binding, e.g. by adding low concentrations of imidazole, and the properties of various fluorophores have been explored previously.

Other approaches employing repetitive labeling with multiple target structures have been reported before. Tam et al. report chemical bleaching with sodium borohydride treatment of samples after imaging allows for multiplexed imaging. The authors compared labeling densities after imaging and found them to be in the same range as the background labeling density. Compared with SERI, this approach utilizes treatments that may interfere with the structural integrity of the sample. Valley et al. performed multi-color imaging by sequential labeling, chemical bleaching, photodestruction and relabeling of different cellular targets. The authors report that following photodestruction, only 0.18% of the originally present fluorophores are active. However, this labeling protocol was so far used for multiplexing, but could in principle also be used for repetitive labeling of a same structure. Elution and restaining with antibodies was also introduced for multi-target super-resolution imaging, and could equally be used with the SERI approach. PAINT and PAINT-like approaches also allow for high labeling densities, for imaging of large three-dimensional volumes as well as of multiple targets. The recently introduced FRET-PAINT is a powerful imaging approach that allows for low-background imaging with high labeling densities and similar three-dimensional imaging capabilities as SERI.

In conclusion, we introduce a small labeling pair that can be used for super-resolution imaging with increased image contrast and for whole-cell 3D imaging. We believe that the development of novel labels that allow specific binding and controlled elution will be beneficial, and could further improve and extend this imaging approach.

**Materials and Methods**

**Cell Culture.** U2OS cells (CLS Cell Line Services, Eppelheim, Germany) were maintained in DMEM (Gibco) with 4.5 g/L glucose (Gibco), supplied with 10% FCS (Gibco), and stored in a humidified tissue culture incubator at 37 °C and 5% CO₂ atmosphere. Passaging was carried out every 2–3 days using PBS (Sigma-Aldrich) and 0.5% trypsin/PBS (GE Healthcare). Transfection was performed using 18 mM of branched PEI with a DNA/PEI ratio of 1:3, following the manufacturer’s instructions. Cells were seeded into a glass-bottom dish with a number of 2 × 10⁴ cells per well and subsequently transfected with 0.2 µg plasmid DNA His_{10}-mEGFP-LaminA per well. After 48 h, cells were chemically fixed with 4% PFA for 30 min.

**Staining His_{10}-mEGFP-LaminA with trisNTA-AlexaFluor647.** After 24 to 48 h, transfected U2OS cells grown on glass bottom dishes were chemically fixed with 4% formaldehyde in PBS for 30 min at room temperature. Cells were permeabilized with 0.1% (v/v) Triton X-100 (Sigma-Aldrich) in PBS for 20 min. Cells were
Figure 3. 3D large volume imaging of laminA-His_{10}-eGFP. (a) Schematic depiction of imaging an entire cell (stippled line), here mostly represented by the nucleus (continuous line), with SERI. The focus is shifted after a defined number of frames during a single imaging cycle to cover the entire volume. Thereafter the cells are treated with elution buffer, re-stained and imaged again. The stippled line denotes the assumed contours of the cell. (b–e) Four different single imaging rounds of a cell transfected with His_{10}-mEGFP-LaminA. Note that measurements 1 (b) and 3 (d) were done in top-down direction while measurements 2 (c) and 4 (e) were done in reverse direction, thereby revealing the bleaching-induced decrease of the number of localizations in the direction of image acquisition (indicated by color code for z position). (f) Overlay of all four single imaging rounds. The scale bars in (b,f) correspond to 5µm of the bottom side of the cell. The scale bar in (b) applies to panels (b–e). (g) xz-projection of a 500 nm thick section. The color bar ranges from 0µm (red) to 4µm (blue). (h) Intensity cross section of the lamin border. (i) Distribution of localizations over the z-positions of the nucleus of each measurement and of the merged measurements. The merged measurement demonstrates an enhanced labeling density over the z-range in comparison to single measurements. Similar results were obtained in a total of N = 4 cells.
Supplemental Fig. 1. available from the corresponding author on reasonable request.

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Author Contributions
F.H., T.K., V.V. designed the study. A.K. and R.W. performed unpublished experiments. A.K., R.W. and R.T. provided labeling protocols, materials, and reagents. F.H., M.K., and V.V. performed experiments and data analysis. F.H., M.H., M.K., R.T., T.K., and V.V. wrote the manuscript.

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