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Live-cell protein labelling with nanometre precision by cell squeezing

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Live-cell labelling techniques to visualize proteins with minimal disturbance are important; however, the currently available methods are limited in their labelling efficiency, specificity and cell permeability. We describe high-throughput protein labelling facilitated by minimalistic probes delivered to mammalian cells by microfluidic cell squeezing. High-affinity and target-specific tracing of proteins in various subcellular compartments is demonstrated, culminating in photoinduced labelling within live cells. Both the fine-tuned delivery of subnanomolar concentrations and the minimal size of the probe allow for live-cell super-resolution imaging with very low background and nanometre precision. This method is fast in probe delivery (~1,000,000 cells per second), versatile across cell types and can be readily transferred to a multitude of proteins. Moreover, the technique succeeds in combination with well-established methods to gain multiplexed labelling and has demonstrated potential to precisely trace target proteins, in live mammalian cells, by super-resolution microscopy.
Direct observation of intracellular processes has the potential to yield insight into fundamental biological pathways and disease mechanisms. Several techniques have been developed to enable high-resolution imaging of living cells; yet, the limited ability to trace intracellular components has hindered progress. Hence, two of the persistent challenges are probe design and cellular delivery with minimal toxicity, pivotal for advances in live-cell imaging technologies. Here we describe an efficient approach to tag and image intracellular components in live mammalian cells. Using the microfluidic cell squeezing platform to deliver small fluorescent tris-N-nitrilotriacetic acid (trisNTA) probes (~1 kDa), we demonstrate highly efficient, minimally disruptive, light-triggered tracing of native proteins and the subsequent super-resolution imaging of live-cell phenomena.

Live-cell microscopy contributed significant knowledge of dynamic processes such as protein trafficking and single-molecule localization-based imaging techniques visualize proteins with high-resolution information (≤50 nm)\(^1\,2\). All fluorescent imaging techniques require protocols to introduce the label with the need to minimize its influence on the system. Fluorescent proteins, self-labeling tags\(^3\)\textendash6 or labelling by enzymatic methods\(^7\,8\) can interfere with protein function, assembly or dynamics. Bulky fusion proteins (>20 kDa) entail the risk of steric hindrance and functional perturbations, whereas smaller tags (for example, tetracysteine tag) deal with unspecific interactions or require additional experimental steps\(^9\) and optimized flanking sequences for each protein target\(^9\).

Although synthetic fluorophores have enhanced photostability, quantum yield, spectral range and localization precision, it is difficult to introduce such probes to the cytosolic environment using existing delivery technologies. On the one hand, current transduction strategies such as delivery by cell-penetrating peptides (CPPs), electroporation and so on are suboptimal, suffering from poor and endosomal uptake, rapid degradation by extracellular and endosomal proteases, low in vivo efficiency or elaborated chemical synthesis. On the other hand, antibody-based labelling approaches, for example, are limited to chemically arrested (fixed) cells and the availability of specific antibodies for a protein target. Owing to the described limitations of existing labelling and transduction technologies, there is a persistent demand for techniques enabling high-throughput in-cell labelling by minimal tags that are conductive to high-resolution and super-resolution microscopy.

Here we demonstrate robust in-cell targeting of native proteins using a labelled multivalent chelator head trisNTA\(^10\) and a genetically encoded oligohistidine sequence (Fig. 1a). trisNTA site specifically recognizes His\(_6\)\textendash10-tagged proteins in the (sub)nanomolar range (K\(_d\) of 0.1–10 nM) even in the crowded cellular environment\(^11\). The minimal size of the tag and the molecular probe allows direct targeting with nanometre precision at subnanomolar concentrations as required for single-molecule localization-based imaging techniques\(^12\,12\,13\) with no impact on intracellular trafficking or demand for additional cofactors affecting endogenous processes. We simplified efficient transfer of the trisNTA probe into living cells by cell squeezing\(^14\), combining precisely controlled cytosolic delivery with high specificity and low cytotoxicity. Briefly, transient cell permeabilization is achieved by rapid viscoelastic deformation of cells as they pass through micrometre-scale constrictions. This facilitates fast uptake of probes into the cytosol before cell-intrinsic repair mechanisms kick in\(^15\).

**Results**

**High-affinity protein labelling at subnanomolar concentration.** We first investigated the specificity of the trisNTA/His tag targeting in chemically arrested cells. To evaluate precise localization, different proteins resident at distinct subcellular compartments were selected: (i) the transporter associated with antigen processing (TAP) in the membrane of the endoplasmic reticulum\(^16\); (ii) histone 2B (H2B) in the nucleus; and (iii) Lamin A at the nuclear envelope. All proteins of interest (POIs) were fused to a His\(_6\) tag and a fluorescent protein (TAP\(^1\)\textsuperscript{1}\textsuperscript{m}Venus-His\(_{10}\), H2B\(_{m}\)Venus-His\(_{10}\) and His\(_{10}\)-mEGFP\(_{17}\)Lamin A) for specific targeting and co-localization studies, respectively. For sensitive detection, trisNTA was covalently coupled to different fluorescent dyes (trisNTA\(^1\), I = Alexa488, ATTO655, ATTO647N, Alexa647 and ATTO655). Mammalian cells were transiently transfected with the corresponding target genes. His-tagged proteins were specifically stained by trisNTA\(^1\) with excellent co-localization and signal-to-noise ratio (Pearson’s coefficients between 0.90 and 0.96), using confocal laser scanning microscopy (CLSM; Fig. 1b and Supplementary Fig. 1). Strikingly, even at 200 pM of trisNTA\(^1\), His-tagged proteins were labelled with high specificity (Supplementary Fig. 2). By analysing a variety of fluorescent dyes, we noticed that trisNTA\(_{m}\)Venus-His\(_{10}\) labelling produced a higher background compared with trisNTA\(_{m}\)Venus-His\(_{10}\), trisNTA\(_{m}\)Alexa488 or trisNTA\(_{m}\)ATTO655 (Supplementary Fig. 3). This was assigned to unspecific binding of the ATTO655 dye. Moreover, the superposition of both fluorescence intensity profiles reflects an excellent correlation between the POI expression level and the labelling density of trisNTA\(_{m}\)Alexa488 (Fig. 1c,d, Pearson’s coefficient r = 0.95). Notably, using nanomolar concentrations, trisNTA\(^1\) labelling is significantly more efficient within 30 min than SNAP\(_{m}\)-tag labelling (Supplementary Fig. 4). In contrast, mammalian cells expressing H2B lacking a His tag showed neither trisNTA\(^1\) labelling nor unspecific staining (Supplementary Fig. 5). In conclusion, trisNTA\(^1\) targeting at subnanomolar concentrations is highly specific to trace His-tagged proteins. To exploit these benefits further, we combined trisNTA\(^1\) with well-established labelling methods for multiplexed protein modification. Specific trisNTA\(_{m}\)Alexa488 labelling of His\(_{10}\)LaminA (Fig. 1e, green) was successfully achieved in combination with SNAP\(_{m}\)-tag labelling of H2B (magenta) and antibody labelling of tubulin (red), as well as the lysosomal-associated membrane protein 1 (blue). Thus, the ultra-small interaction pair complements the toolbox of well-established labelling techniques and the nanomolar concentrations perform various avenues in multiplexed labelling.

**High-throughput live-cell labelling within mammalian cells.** Encouraged by these observations, we aimed at protein labelling in living cells. To transfer trisNTA\(^1\) into cells, we applied microfluidic cell squeezing (Fig. 2a). As the trisNTA\(^1\) probes are chemically diverse relating to the used fluorophores, common transduction strategies are unlikely to efficiently deliver nanomolar concentrations of trisNTA\(^1\) into mammalian cells. Specifically, mammalian cells were mechanically pushed (‘squeezed’) through micrometre constrictions at elevated pressure of 30 psi. This approach allows for high cell survival (>90%) and efficient uptake of trisNTA\(^1\) (up to 80%; Supplementary Figs 6 and 7). Energy-dependent endocytosis, often observed at cargo transfer with supercharged molecules (Supplementary Fig. 8) or low concentrations of CPPs (Supplementary Fig. 9\(^17\)), were prevented by performing cell squeezing at 4°C (Supplementary Fig. 10).

By squeezing TAP\(^1\)\textsuperscript{1}\textsuperscript{m}Venus-His\(_{10}\)-transfected HeLa cells in the presence of trisNTA\(^1\) (100 nM), we achieved a high-throughput delivery and a high-density labelling, illustrated by an excellent co-localization between both reporter molecules (Fig. 2b). We noticed that both probe delivery by cell squeezing and protein labelling are highly reproducible (n > 20). To quantify the
with the subcellular localization in chemically arrested cells. Notably, live-cell labelling is independent of cell types, for example, HeLa, HeLa Kyoto, Chinese hamster ovary (CHO-K1) or human embryonic kidney 293 cells (Figs 1b and 2d). Beyond that, trisNTA\textsuperscript{f} labelling after removal of the bulky fluorescent protein fully exploited the small size of the lock-and-key element and confirmed again specific labelling in living cells with minimal perturbation (Supplementary Fig. 12). The specificity was validated in cells transfected with H2BEGFP lacking a His tag and trisNTA\textsuperscript{A} or of free Alexa647 dye showed no labelling in TAP1\textsuperscript{mVenus-His\textsuperscript{10}}-transfected cells (Supplementary Figs 14 and 15). In contrast, trisNTA\textsuperscript{A} (Fig. 2 and Supplementary Fig. 14) and trisNTA\textsuperscript{A} (Supplementary Fig. 16) clearly stain TAP1 mVenus-His\textsuperscript{10} at the endoplasmic reticulum membrane after cell squeezing. Noticeably, cell viability of trisNTA\textsuperscript{f}-transduced cells was negligibly affected 1 and 24 h after labelling. Similar concentrations of unbound nickel ions inside mammalian cells had no significant toxic effects (Supplementary Fig. 7). In contrast, electroporation entailed more than twofold increased toxicity.
compared with squeezing (Supplementary Figs 7 and 11). Collectively, nanomolar delivery of trisNTA
\(^{4}\) fully realized the potential of in-cell protein manipulation with minimal perturbation and modification rates exceeding common approaches.

After successful in-cell labelling of different His-tagged proteins, we aimed for in vivo multiplexed labelling by combining trisNTA
\(^{4}\) with well-established labelling methods. By trisNTA
\(^{Alexa647}\) delivery via squeezing and subsequent SNAP
\(^{4}\)-tag labelling, we achieved specific and distinct targeting of His
\(^{10}\) LaminA in the presence of two different SNAP
\(^{4}\)-tagged proteins in live cells (Fig. 3a and Supplementary Fig. 17). Hence, trisNTA
\(^{4}\) complements the toolbox for in vivo multiplexed labelling, offering minimal disturbance due to its small size and simultaneously using low nanomolar concentrations.

We next determined the minimal reporter concentration required for specific live-cell labelling. Well-resolved images of TAPI
\(^{Venus-His^{10}}\) were obtained even at 1 nM of trisNTA
\(^{Alexa647}\) (Fig. 3b). Based on previous observations, approximately one-third of the cargo provided during squeezing is the effective intracellular concentration
\(^{14}\). Thus, the estimated cytotoxic concentration of trisNTA
\(^{4}\) further corroborates the high target sensitivity at subnanomolar concentrations (\(~300\ pM\)). These results are in line with the detection limit of \(~200\ pM\) trisNTA
\(^{Alexa647}\) in chemically arrested cells (Supplementary Fig. 2). Hence, this enables the precise adjustment of the effective, intracellular trisNTA
\(^{4}\) concentration to improve the signal-to-background ratio, hardly realized by alternative approaches at nanomolar probe concentrations (for example, CPPs, SNAP, CLIP and Halo tag; Supplementary Figs 4 and 9)
\(^{14,18}\), and circumvents endocytic uptake observed with supercharged proteins at similar nanomolar concentrations (Supplementary Fig. 8)
\(^{19}\).

**In-cell protein modification with nanometre precision.** Incited by this observation, we aimed at temporal and spatial control of protein tracing by light, which depends on low probe concentrations for high signal-to-background ratios. Using photoactivatable trisNTA
\(^{4}\) (PA-trisNTA
\(^{ATTO655}\))
\(^{20}\) for dynamic cellular imaging on demand, light-activated in vivo labelling of His
\(^{10}\)-mEGFP-
\(^{Lamin A (Chinese hamster ovary (CHO-K1)) or H2BmVenus-His^{10} (HEK293) by squeezing. High target specificity of...}}}$

**Discussion**

We established a high-throughput method for protein labelling inside living cells using a minimalistic lock-and-key probe. Our method is versatile in the choice of the molecular probe, cell type and the subcellular localization of the POIs, a persistent challenge in live-cell analysis. The high-affinity trisNTA/His tag interaction pair enables fast labelling (\(~10\ min\)) at subnanomolar concentrations with tunable labelling density and flexibility of cell-impermeable organic fluorophores. Compared with carrier-mediated transport by CPPs
\(^{11,18}\), delivery of 1,000-fold...}
lower concentrations (nM versus μM) effectively decreases the fluorescence background. Furthermore, high-throughput analysis with up to 1,000,000 cells per second can be achieved in contrast to microinjection. In addition, trisNTA delivery via squeezing avoids endocytic cargo uptake, frequently observed with low CPP concentrations and supercharged molecules, offering decreased toxicity and a >30-fold higher efficiency compared with electroporation. The minimal probe complements the toolbox of well-established labelling techniques such as self-labelling enzymes and can be combined with the latter to achieve distinct labelling of different proteins in fixed as well as in living cells. Moreover, in situ photoactivation of PA-trisNTA allows labelling at defined time points, to trace proteins for dynamic cellular function. The speed, flexibility and efficiency for high-throughput live-cell targeting of proteins even if assembled in stable and transient macromolecular complexes. This study is exploited via one of the smallest high-affinity lock-and-key recognition pairs known so far and allows even multiple cargos to be delivered simultaneously, displaying diverse chemical properties. The quantity of cargo for in-cell manipulation can be precisely tuned and the biological output can in turn be fine-tuned. As the affinity tag is widely used in life sciences and our delivery platform is broadly applicable across cell types, this live-cell labelling method could potentially be implemented across numerous cell-impermeable probes and prodrugs, as well as translated to difficult cell lines including patient-derived cells and embryonic stem cells, providing the opportunity to use these cells for advanced microscopy techniques and live-cell analysis.

Methods

Plasmid construction. The H2B construct H2BmVenus-His10 was generated by consecutive insertion of H2B and mVenus-His10 into pCDNA3.1(+)(Life Technologies). mVenus-His10 was PCR amplified using Phusion High-Fidelity DNA Polymerase (Fermentas) and the primer pair forward (fw) 5’-GCGGCCGCGTGAGCAAGGGCGAGGAGCTGTTCA-3’ and reverse (rev) 5’-GCGGGCCTCTAGATTAGTGTTGGTGATTGATGG-3’ (Xbal restriction site underlined) and cloned into the pCDNA3.1(+) plasmid for His-tagged POIs in living cells. Subsequently, H2B was amplified using the primer pair rev 5’-CCAGAGCCAGCGAAGTCTGCTCCCGC-3’ (Acc65I restriction site underlined) and reverse (rev) 5’-GCGGGCCTCTAGATTAGTGTTGGTGATTGATGG-3’ (Xbal restriction site underlined), and cloned into the pCDNA3.1(+) plasmid, amino terminally of mVenus-His10. A sense primer 5’-CTCGAGGAGCCAGCGAAGTCTGCTCCCGC-3’ (NotI restriction site underlined), and reverse (rev) 5’-GCGGGCCTCTAGATTAGTGTTGGTGATTGATGG-3’ (Xbal restriction site underlined) and rev 5’-GCGGGCGCCGCGCTCCGGG (EcoRV restriction site underlined, stop codon bold), and cloned into the pCDNA3.1(+) plasmid using the restriction enzyme HindIII (Fermentas). As a template for the amplification of H2B, the plasmid pEGFP-N1 containing the human H2B sequence (plasmid 11680, Addgene) was used, which also served as template for the amplification of H2B, the plasmid pEGFP-N1 containing the human H2B sequence (plasmid 11680, Addgene) was used, which also served as template for the amplification of H2B. As a template for the amplification of the full-length H2B, the plasmid NTAAlexa647 was used containing the indicated restriction enzymes (Fermentas). mVenus-His10 was PCR amplified using Phusion High-Fidelity DNA Polymerase (Fermentas) and the primer pair forward (fw) 5’-GCGGCCGCGTGAGCAAGGGCGAGGAGCTGTTCA-3’ and reverse (rev) 5’-GCGGGCCTCTAGATTAGTGTTGGTGATTGATGG-3’ (Xbal restriction site underlined) and cloned into the pCDNA3.1(+) plasmid, amino terminally of mVenus-His10.
CATGGAGACC-3′ (BamHI restriction site underlined, start codon bold, His10-tag italic) and AGCTGCTGCTGGGCGTCTTG-3′ (NotI restriction site underlined, stop codon bold). Indicated restriction sites were used to insert this PCR product into the pcDNA3.1 (+) vector. The pSNAP-H2Bz and pSNAP- CoxA plasmids (New England Biolabs) were used for SNAP-tag labelling. In addition, a planimetric alignment for the core domain of TAPI, tagged with mVenus-His10 (TAPImVenus-His10) was used as previously described23.

Cell culture and transfection. HeLa cells, HeLa Kyoto cells, Chinese hamster ovary (CHO-K1) cells and human embryonic kidney 293 cells were maintained in DMEM medium with 4.5 g/l glucose (Gibco), supplied with 10% (v/v) FCS (Gibco) in T75 cell culture flasks (Greiner). Every 2–3 days, cells were passaged using PBS (Sigma-Aldrich) and 0.05% trypsin/0.02% EDTA/PBS (GE Healthcare). All cell lines were cultivated in a humidified tissue culture incubator at 37 °C and 5% CO2. Mycoplasma contamination tests were carried out regularly, following the guidelines described24. Transient transfection was performed with Lipofectamine 2000 (Life Technologies), following the manufacturer’s instructions. For fixation and staining, 2 × 105 cells per well were seeded into eight wells on cover glass II slides (Sarstedt) and transfected with 0.2 μg DNA per well. For squeezing experiments, 8 × 105 cells were seeded into six-well cell culture plates (Greiner) and transfected with 2 μg DNA per well. After transfection, cells were incubated 12–48 h at 37 °C and 5% CO2 until experiments were performed.

Cell viability test. To analyse cell viability after squeezing, the Sytox Blue Dead Cell stain (Life Technologies) was used to stain cells with a permeable plasma membrane. Cells were squeezed in the presence of 100 nM trisNTAf (49–57) n = 100 nM Alexa647, 500 nM dye or 500 nM NiCl2, followed by fixation with 1 μM of Sytox Blue Dead Cell Stain (20 min, room temperature (RT)) at different time points. Cell viability analysis was performed using the Attune flow cytometer (Life Technologies) and data were processed using FlowJo 7.6.5 (Tree Star Inc.). Before detaching the cells, the supernatant was collected to avoid altering the results by removing dead cells during washing. Identical Sytox Blue Dead Cell Staining was conducted with cells after electroporation (described above), followed by flow cytometry analysis of cell viability and uptake of trisNTAf9,36. All experiments were performed in triplicates and error bars indicate the s.d.

Confocal imaging. Imaging was performed using the confocal laser scanning TCS SP5 microscope (Leica) and a Plan-Appochromat 63×/1.4 Oil differential interference contrast objective. Images were acquired sequentially to avoid cross-talk. The following laser lines were used for excitation: 405 nm (diode laser) for ATTO647N, Alexa647 and ATTO655. For image analysis, 565 nm (diode-pumped solid-state laser) for ATTO565 and TMR-Star; and 633 nm (helium–neon laser) for O6-benzyl guanineAlexa647 (New England Biolabs) 24 h after transfection. After washing with PBS, cells were fixed with 4% formaldehyde/PBS and incubated with 10 or 100 nM of benzyl guanineAlexa647 for 30 min at 37 °C. After three washing steps with 0.1% Triton X-100/0.5% BSA/PBS and two washing steps with PBS, imaging was conducted by CLSM. In case of combined trisNTAf- and SNAP-tag labelling, SNAP-tag labelling was performed first, followed by trisNTAf labelling as described above.

trisNTAf delivery by cell squeezing. Squeezing was performed using a chip with constrictions of 7 μm in diameter and 10 μm in length (CellSqueeze 10-7X1, SQBiotech), if not otherwise stated. In all microfluidic experiments, a cell density of 1.5 × 105 cells per ml in 10% (v/v) FCS/PBS were squeezed through the chip at a pressure of 30 psi. Transduction was conducted at 4 °C, to block cargo uptake by endocytosis. During squeezing, the following cargo concentrations were used: 0.1–100 nM trisNTAf (f as described above) and 500 nM SNAPf-tag labelling, cells were first squeezed in the presence of 100 nM trisNTAf and 3 μM SNAPf-Tag labelling as described above.

HeLa Kyoto cells transfected with H2BSNAPf (f in 1% (w/v) BSA/PBS were squeezed through the chip at 0.5 psi. Transduction was conducted at 4 °C, to block cargo uptake by endocytosis. After washing with PBS, cells were fixed with 4% formaldehyde/PBS (10 min, RT), washed twice with PBS and subsequently permeabilized using 0.1% Triton X-100/100/0.5% BSA/PBS and incubated with 10 or 100 nM of benzyl guanineAlexa647 for 30 min at 37 °C. After three washing steps with 0.1% Triton X-100/0.5% BSA/PBS and two washing steps with PBS, imaging was conducted by CLSM. In case of combined trisNTAf- and SNAP-tag labelling, SNAP-tag labelling was performed first, followed by trisNTAf labelling as described above.

Proteins were purified via immobilized metal ion chromatography using Ni Sepharose 6 Fast Flow (GE Healthcare). Elution was performed with 500 mM imidazole before desalting of the eluted protein was conducted with PD-10 desalting columns (GE Healthcare).
laser for 10 s. Imaging of both channels (mEGFP and ATTO655) was performed before and directly after photoactivation.

References


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Author contributions

A.K. designed and performed the cell squeezing and labelling experiments. A.S. determined the squeezing efficiency. A.S., R.L. and K.F.J. designed and provided the microfluidic devices. A.R. and M.H. performed the sSTORM imaging and analysis. A.K., R.W. and R.T. wrote the manuscript and analysed the data. R.W. and R.T. conceived the ideas and directed the work.

Additional information

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