Simple generation of cleavable labels for multiplexed imaging

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Supplementary information

Materials & Methods

Antibody labeling

We labeled five antibodies (α-Tubulin antibody (MA1-80017, ThermoFisher Scientific), β-Actin antibody (ab6276, Abcam), Vimentin antibody (ab24525, Abcam), KI-67 (55603, BD bioscience) and CD44 antibody (MCA2726, Biorad)). Residual azides from the antibody storage buffer were removed in two consecutive buffer exchanges using Zeba spin columns (7,000 Da MWCO, ThermoFisher Scientific) in labeling buffer (PBS at pH 6) according to the manufacturer's protocol. This slightly acidic pH was chosen in order to avoid premature cleavage of the S-S bond in the linker. The antibodies were then concentrated to a concentration of >1 mg/ml using spin columns (Amicon Ultra 0.5 mL, 100k MWCO). A 50-fold molar excess of DBCO-SS-NHS ester (Conju-Probe, USA) and 150-fold molar excess of azide AF555 (Jena Bioscience) (Supplementary figure S6) were added in a total volume of 50 µl. For conventional labeling, 50-fold molar excess of NHS-AF555 (Jena Bioscience) was added. After overnight incubation at 4°C, the reaction was stopped by addition of 1.5M Tris. Unreacted dye and linker were removed by two consecutive buffer exchanges on the Zeba spin columns to PBS at pH 6. The labeling efficiency was verified using a Nanodrop spectrophotometer (ThermoFisher Scientific).

Cell fixation and staining

15000 U2OS cells (ATCC-HTB-96) cells were seeded on 1.5H glass bottom 8 Well µSlides (Ibidi). 24 hours post seeding, cells were fixed. Briefly, cells were incubated for 90s with pre-extraction buffer (0.4 % v/v glutaraldehyde and 0,25 % v/v Triton X-100 in 1X PBS (pH 7.2)). After removal of pre-extraction buffer, cells were incubated for 15 minutes in enhanced microtubule fixative (3 % glutaraldehyde in 1X PBS (pH 7.2)). Cells were washed four times (1x 20s, 3x 5min) in 0.02 % Tween20 and 1mM EDTA in 1X PBS (pH 7.2) and then incubated in blocking buffer (3 % w/v BSA, 0.02 % Tween20 and 0.05mg/ml $NaBH_4$ in 1X PBS (pH 7.2)) O/N at 4 degrees. We then incubated the primary antibody (20 µg/ml for α-Tubulin cleavable fluorescent antibody , 20 µg/ml for conventionally labeled anti α-Tubulin, 5 µg/ml for β-actin antibody containing the cleavable linker and 40 µg/ml for the vimentin antibodies containing the cleavable linker diluted in 3% BSA in 1x PBS (pH 7.2)) for 1 hour at room temperature. Actin filament staining was performed incubation of 165 nM phalloidin ATTO488 in antibody dilution buffer for 30 minutes at room temperature. For staining KI-67, 15000 HeLa cells (ATCC-CCL-2) were seeded on 1.5H glass bottom 8 Well µSlides (Ibidi) and fixed in formaldehyde/methanol. Cells were incubated for 15 minutes in 4% formaldehyde on ice, followed by 5 min incubation with methanol on -20 degrees. Afterwards, they were blocked in blocking buffer, followed by staining with 10 µg/ml for α-Tubulin cleavable fluorescent

antibody, 50 µg/ml KI-67 cleavable fluorescent antibody and 10 µg/ml CD44 cleavable antibody.

Image acquisition

Imaging was performed on a Nikon Eclipse Ti-2 Inverted Microscope (Minato City, Japan) equipped with a 1.4 NA oil immersion objective (x100 CFI Apochromat Total Internal Reflection Fluorescence) and a 4-band cube in TIRF-illumination. A 561 nm or a 488 nm laser (Oxxius, Lannion, France) was used for excitation. Images were acquired on a pco.edge 4.2 camera (PCO, Kelheim, Germany) with an exposure time of 100 ms and projected pixel size of 118.18 nm.

Dye removal and restaining

Destaining was performed using 30 minutes of DTT treatment (50 mM DTT in 1X PBS (pH 7.2)) at room temperature, after which cells were washed three times using PBS (3x 5min) by on-stage pipetting. Subsequent staining was performed as described above, with the destaining-restaining procedure repeated as needed.

Live cell staining, imaging and destaining

15000 HeLa cells (ATCC-CCL-2) cells were seeded on 1.5H glass bottom 8 Well µSlides (Ibidi). 24 hours post seeding, cells were washed incubated in Fluorobrite medium (Thermo Fisher) with 10 µg/ml CD44 cleavable antibody for 1 hour. Imaging was performed on an Olympus IX71 inverted microscope equipped with a Spectra X Light Engine (Lumencor), a 10 × UplanSApo objective (Olympus), a ZT561RDC dichroic mirror and HQ572lp emission filter (both Chroma), and an ORCA-Flash4.0 LT+ sCMOS camera (Hamamatsu). Destaining was performed using a 60 minutes DTT treatment (50 mM DTT in Fluorobrite)

Tissue samples

Tissue sections (3 µm) were prepared from FFPE human glioblastoma samples (S59804 - S61081 - S62248). Following dewaxing, antigen retrieval was performed using PT link (Agilent) using 10 mM EDTA in Tris-buffer pH 8. Immunofluorescence detection of KI-67, alpha-Tubulin and CD44 was performed in a cyclic manner. Sections were incubated with primary antibodies for 1 hour at room temperature, followed by 5 minute treatment with DAPI. Images were acquired using the PhenoImager (Akoya Biosciences). Subsequently, fluorophores were cleaved and removed with 50 mM Dithiothreitol (R0861, Fisher Scientific) for 30 minutes at room temperature. Following antibody cleavage, the following primary antibody was incubated using a cyclic approach until all markers were stained and imaged.

Data analysis

Images were analysed using Igor Pro (Wavemetrics) and FIJI. To obtain quantitative data, we followed two different approaches. First, images were registered. Average fluorescence intensity was obtained after background subtraction with the prestained image. Data was normalized to the pre-DTT values. Peak to valley fluorescence was determined using a line ROI through clearly distinct fibers. Peaks and valleys intensities were averaged, after which peak values were subtracted with valley values. Subtracted values where then normalized to pre-DTT values.

Supplementary figures

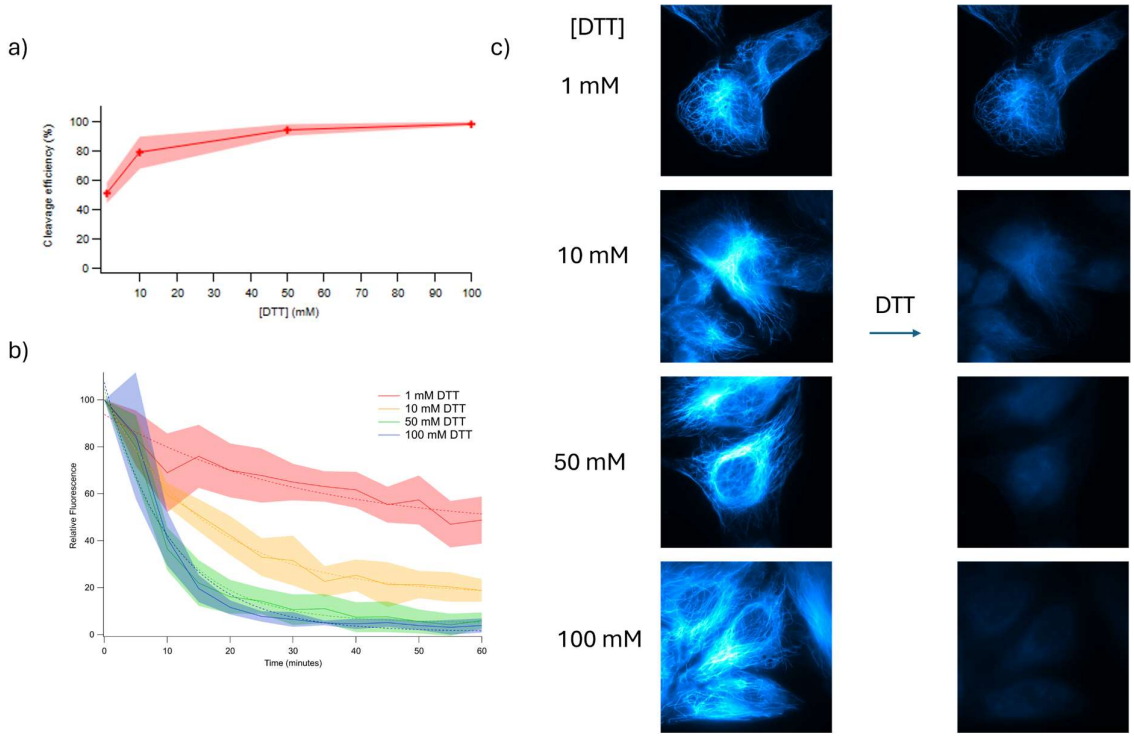

Figure S1. Titration of anti α-Tubulin-antibody-SS-AF555 cleavage by DTT. (a) End-point cleavage efficiency of DBCO-SS-NHS linkers with 1 mM , 10mM, 50 mM and 100 mM of DTT after 60 minutes. (b) Time trace peak-to-valley intensity after DTT treatment. (c) Representative images before and after DTT treatment at different concentrations.

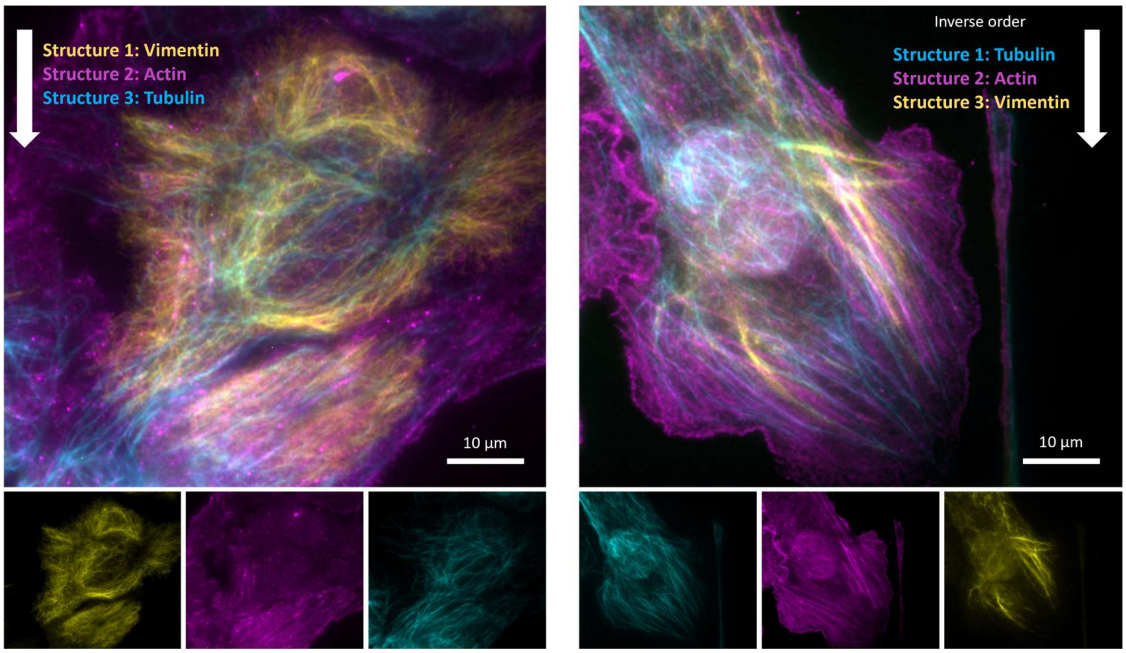

Figure S2. Representative images showing sequential staining-imaging-cleaving with three antibodies labeled with AF555 in two different orders.

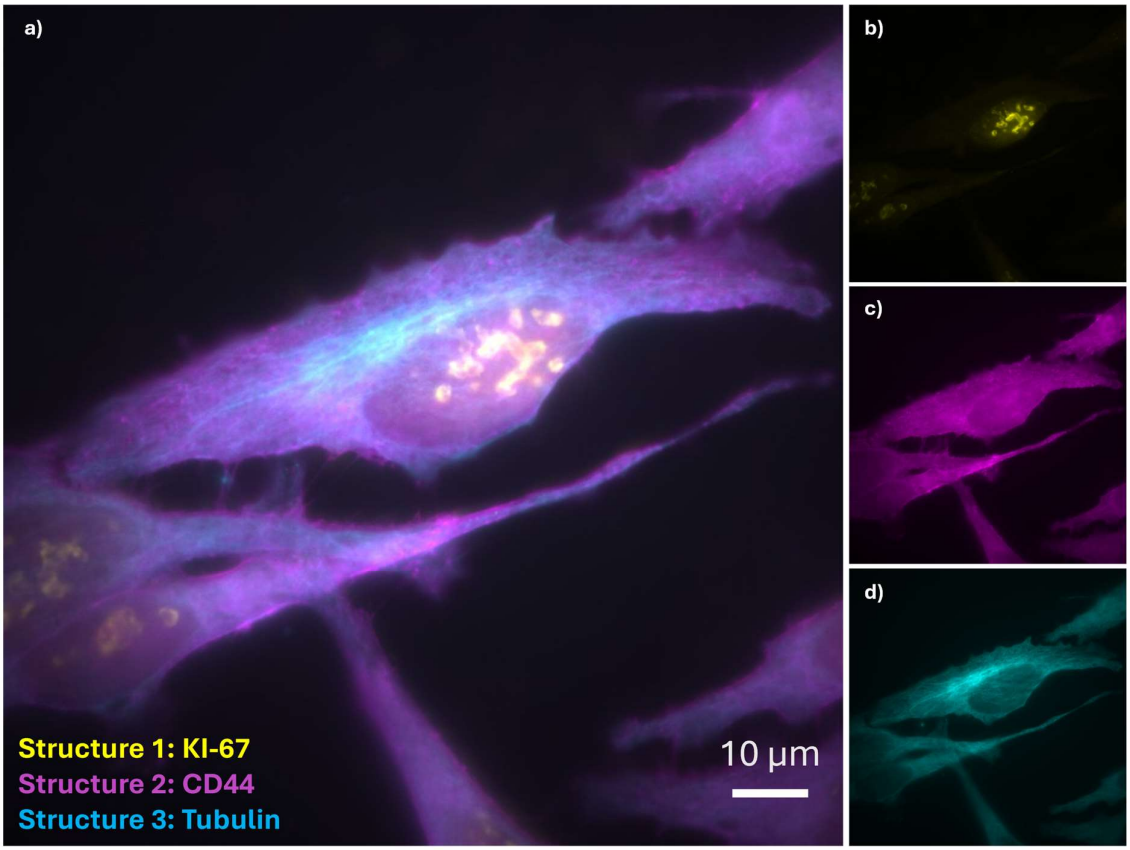

Figure S3. Cyclic staining proof-of-concept. (a) Sequential staining of Hela cells with three AF555-labeled antibodies using the cyclic immunostaining protocol. Yellow: KI-67, Magenta: CD44, Cyan: α-Tubulin. (b-d) Monochrome panels for each structure.

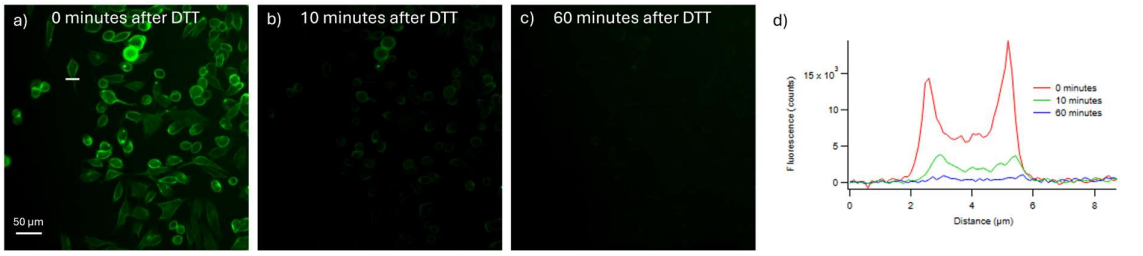

Figure S4. Live cell imaging. (a-c) Hela cells stained with anti-CD44-SS-AF555 at different timepoints after DTT addition. (d) Line plot through a CD44 stained cell at different timepoints.

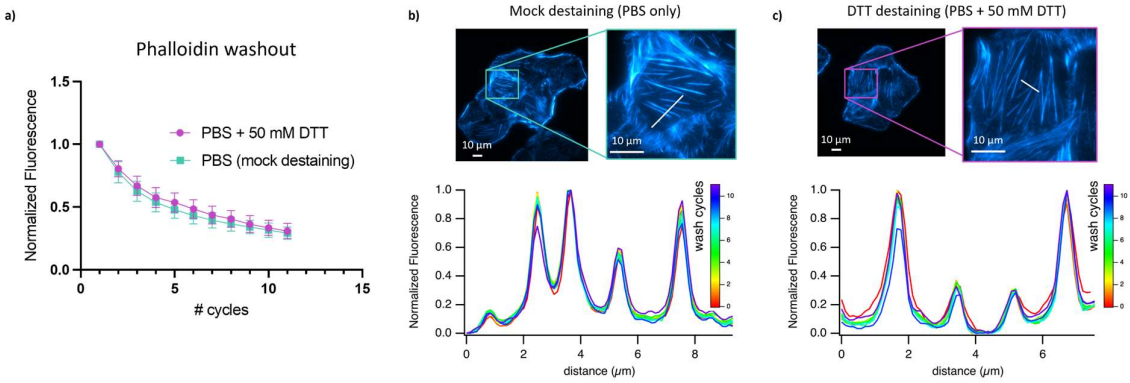

Figure S5. *Structural integrity of actin fibers in repeated destaining cycles. (a) Fluorescence intensities of U2OS cells stained with phalloidin and destained for 10 cycles with PBS containing 50 mM DTT or no DTT (n=20 for each condition, data points on graph are mean values together with the error). (b-c) Lineplot intensities through phalloidin stained fibers followed over 10 destaining rounds with PBS or PBS + 50 mM DTT.*

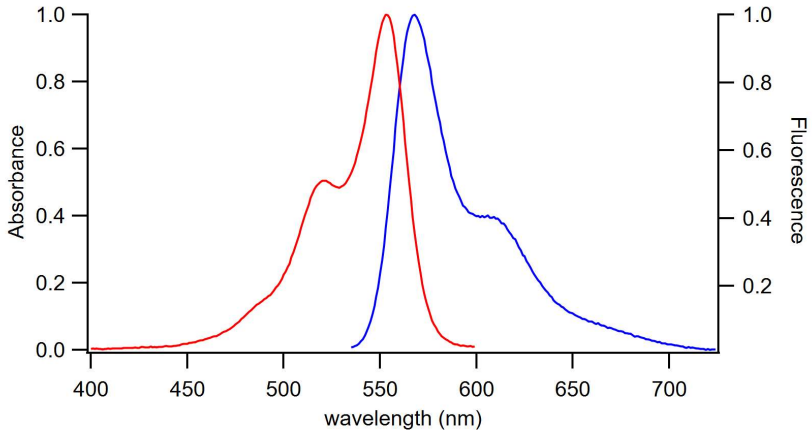

Figure S6. Excitation and emission spectra of AF555.

***Table S1**. Comparison of cleavable linkers for antibody-fluorophore conjugates.*

Method	Scissors	Remaining signal	Incubation time	Linker availability	reference
DBCO-SS-NHS	50 mM DTT	<5%	30 min	commercially available	our method
optoplex	UV light	30%	5 -15 min	organic synthesis	https://doi.org/10.1101/2024.03.18.585511
enzyplex	bdSENP1 protease	<10%	2.5 min - 15 min	custom protein production	https://doi.org/10.1101/2024.03.18.585511
Chemiplex	10 mM TCEP	<15%	15 min	commercially available/ custom peptide production	https://doi.org/10.1101/2024.03.18.585511
FLASH-off	UV light	<10% (extracellular Ab)	<10 s/FOV	organic synthesis	https://doi.org/10.1021/jacs.3c00170
CFA	100 mM TCEP	<5%	30 min	organic synthesis	https://doi.org/10.1002/anie.201611641
Click&Quench (SAFE)	BHQ3-N-tetrazine	<1%	<30 sec	organic synthesis	https://doi.org/10.1038/s41587-022-01339-6
dextran decorated with short ssDNA	DNase I and dextranase	<2%	15 min	organic synthesis	https://doi.org/10.1039/D4CB00007B