Fluorinated Nanobodies for Targeted Molecular Imaging of Biological Samples using Nanoscale Secondary Ion Mass Spectrometry

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1. Materials and General Methods for Chemical Synthesis

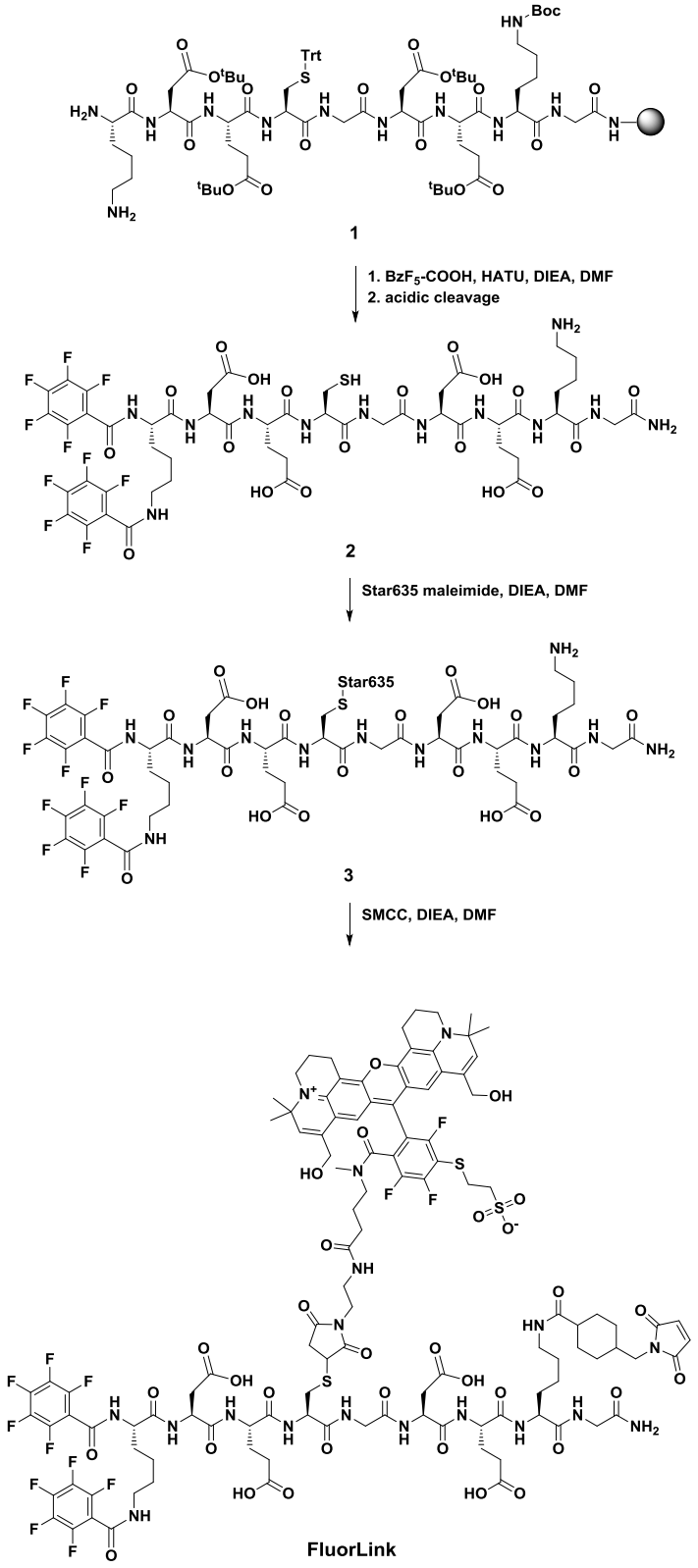
Reagents. Starting materials and reagents were of the highest grade available from commercial sources and were used as delivered. Fmoc-protected amino acids, coupling reagents and Sieber amide resin were purchased from Iris Biotech (Marktredwitz, Germany) and Carl Roth (Karlsruhe, Germany). Star635 maleimide was obtained from Abberior (Göttingen, Germany). Non-conjugated nanobodies FluoTag®-X2 anti-green fluorescent protein (GFP) #1 and #2 (N0304), FluoTag®-X2 anti-mCherry fluorescent protein (mCherry) #1 and #2 (N0404), and FluoTag®-X2 anti-mouse IgG (N1202) were supplied from NanoTag Biotechnologies (Göttingen, Germany). **Solvents.** Anhydrous solvents of extra dry or puriss. absolute grade (over molecular sieves) were obtained from Acros-Organics (Geel, Belgium). Acetonitrile and methanol (MeOH) for HPLC in respective grade and all other solvents of the grade puriss. p.a. were purchased from commercially available sources and were used as supplied. Water for HPLC (milliQ H_2O) and buffers was purified using MilliQ Advantage A10 from Merck Millipore (Darmstadt, Germany). Buffers were degassed by stirring under vacuum and kept under argon atmosphere afterwards. The pH of buffers was adjusted with a pH meter from Hanna Instruments (Vöhringen, Germany) before every use. **Reactions.** Air- and water-sensitive reactions were applied for small scales with a purge-and-refill technique in non-heatable tubes. **Instruments.** Electrospray-ionization (ESI) mass spectra were recorded with a maXis and a micrOTOF spectrometer from Bruker. The values are given as mass per charge (*m/z)*. Microwave-mediated manual solid phase peptide synthesis (SPPS) was realized on a CEM Discover microwave instrument (Kamp-Lintfort, Germany). Reverse-phase high-performance liquid chromatography (HPLC) was performed on a JASCO (Groß-Umstadt, Germany) with a two pump system PU-2080Plus, a multi wavelength detector MD-2010Plus with an analytical or preparative cell, a 3-line degasser DG-2080-5S, and an interface LC-Net II/ADC using a Nucleodur® RP C-18 analytical HPLC column (250 × 4.6 mm, 5 µm) and a C-18 semipreparative HPLC column (250 × 10 mm, 5 µm) from Macherey-Nagel (Düren, Germany). Reverse-phase HPLC runs were recorded on ChromNav as system software and carried out using a linear gradient of 0.1% aq. TFA (solvent A) and CH_3CN/0.1% TFA (solvent B) in 30 min. Flow rates were taken as 1 mL/min for the analytical, and 3 mL/min for the semi-preparative purpose. The UV

absorbance of peptides could be detected at three different wavelengths (215 nm, 254 nm and 280 nm) simultaneously. For the detection of compounds having Star635 fluorophores, its respective major absorption wavelength was used for detection instead of 280 nm. Size-exclusion HPLC was performed on an Äkta pure 25 instrument from GE Healthcare Life Science with internal Unicorn 7.0 software using Superdex 75 Increase 10/300 GL, a column from GE Healthcare Life Science (Amersham, United Kingdom). Isocratic elution with phosphate-buffered saline (PBS, 137 mM NaCl, 2.7 mM KCl, 10 mM Na_2HPO_4, 2 mM KH_2PO_4, pH 7.4) as mobile phase with a flow rate of 0.8 mL/min was applied. The samples were eluted within 1.5 column volume, which corresponds to 23.562 mL and detected by UV-absorbance at 280 nm and 635 nm (fluorophore-conjugated probes). Freeze-drying of compounds from aqueous solutions containing minimal amounts of MeOH or acetonitrile was performed using a Christ-Alpha-2-4 lyophilizer attached to a high vacuum pump and a Christ RCV-2-18 ultracentrifuge (Osterode am Harz, Germany). UV-Spectra for determining the concentration of fluorophore-labeled peptides were recorded with a Thermo Scientific Nanodrop 2000c (Waltham, Massachusetts, USA). Gels for sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) were visualized using an Amersham Imager 600 from GE Healthcare Life Science.

2. Materials and General Methods for Biochemical and Cellular Experiments

Materials. Cell culture reagents and substances were purchased either from Sigma Aldrich (now Merck, Darmstadt, Germany) or AppliChem GmbH (Darmstadt, Germany), unless stated otherwise. The transfection reagent Lipofectamine® 2000, Opti-MEM, and the Click-iT® Cell Reaction Buffer Kit were obtained from Life Technologies (now Thermo Fisher Scientific, Darmstadt, Germany). The rabbit polyclonal anti-PMP70 (ab85550) and the mouse monoclonal anti-Lamin-B2 (ab151735) were purchased from Abcam (Cambridge, UK), whereas the goat anti-rabbit IgG-Alexa Fluor®488 (111-545-144) and the mouse anti-goat IgG (205-005-108) were purchased from Dianova (Hamburg, Germany). The mouse monoclonal antibody anti-GFP (A11120) was obtained from Invitrogen (now Thermofisher Scientific, Karlsruhe, Germany) and the mouse monoclonal anti-α-Tubulin (302 211) from Synaptic Systems (Göttingen, Germany). **Cells.** For the study cell line derived from monkey kidney (COS-7) and **h**uman **e**mbryonic **k**idney HEK293 cells were used. The cells were maintained in culture in Dulbecco's Modified Eagle Medium (DMEM, Thermo Fisher Scientific, Darmstadt, Germany) with 5% fetal calf serum, 2 mM L-glutamine, 60 U/mL penicillin, and 60 U/mL streptomycin**. Constructs.** The plasmids TOM70-mCherry, TOM70-eGFP and Synaptophysin-eGFP were produced in-house and validated via sanger sequencing.

3. Synthesis of FluorLink-nanobodies



Supplementary Scheme S1: Synthesis of FluorLink.

F_5Bz-Lys(F_5Bz)-Asp-Glu-Cys-Gly-Asp-Glu-Lys-Gly-NH_2 (1)

$C_{51}H_{60}F_{10}N_{12}O_{19}S$ [1367.15]

The first step shown in supplementary scheme S1 included the coupling of 1,2,3,4,5-pentafluorobenzoyl groups to H-Lys-Asp(O^tBu)-Glu(O^tBu)-Cys(Trt)-Gly-Asp(O^tBu)-Glu(O^tBu)-Lys(Boc)-Gly-Sieber amide resin (**1**). The synthetic procedure for peptide **1** and the conditions for fluorine coupling were adapted from previous publications.[1,2] The fluorinated compound **2** was obtained as a white solid.

Analytical data: HPLC (C-18, analytical, gradient 20 → 80% B in 30 min): t_R = 14.68 min. *m/z* (ESI) = 695.2 [*M* + H + Na]$^{2+}$, 1367.4 [*M* + H]$^+$. *m/z* (HR-ESI-MS) = calculated: 684.1902 [*M* + 2H]$^{2+}$, 1367.3731 [*M* + H]$^+$, 1389.3551 [*M* + Na]$^+$, found: 684.1897 [*M* + 2H]$^{2+}$, 1367.3738 [*M* + H] $^+$, 1389.3548 [*M* + Na]$^+$.

F_5Bz -Lys(F_5Bz)-Asp-Glu-Cys(Star635)-Gly-Asp-Glu-Lys-Gly-NH_2 (2)

$C_{102}H_{113}F_{13}N_{16}O_{29}S_3$ [2370.27]

Under argon atmosphere, the peptide **2** (1.43 mg, 1.48 µmol, 4.44 eq) was suspended in degassed PBS (pH 7.26, 50 mM sodium phosphate buffer + 100 mM NaCl, 900 µL).[1] Abberior® Star635-maleimide in dry DMF (34.0 µL, 340 µg, 334 µmol, 1.0 eq) was added to the solution. The reaction mixture was stirred for 2.5 h at room temperature (RT) excluding light and stored over afterwards night at +4 °C. The crude product was purified by HPLC. The compound **3** (725 µg, 304 nmol, 91%) was obtained as a blue solid after freeze-drying.

Analytical data: HPLC (C-18, analytical, gradient 30 → 80% B in 30 min): t_R = 15.31–17.01 min. *m/z* (ESI) = 825.2 [*M* + 4Na]$^{3+}$, 1215.4 [*M* + 2Na]$^+$. *m/z* (HR-ESI-MS) = calculated: 810.2260 [*M* + 2Na +H]$^{3+}$, 817.5533 [*M* + 3Na]$^{3+}$, 824.8806 [*M* + 4Na - H]$^{3+}$, 1203.8444 [*M* + Na + H]$^{2+}$, 1214.8354 [*M* + 2Na]$^{2+}$, found: 810.2263 [*M* + 2Na + H]$^{3+}$, 817.5536 [*M* + 3Na]$^{3+}$, 824.8808 [*M* + 4Na + H]$^{3+}$, 1203.8462 [*M* + Na + H]$^{2+}$, 1214.8366 [*M* + 2Na]$^{2+}$.

F_5Bz -Lys(F_5Bz)-Asp-Glu-Cys(Star635)-Gly-Asp-Glu-Lys(MCC)-Gly-NH_2, FluorLink

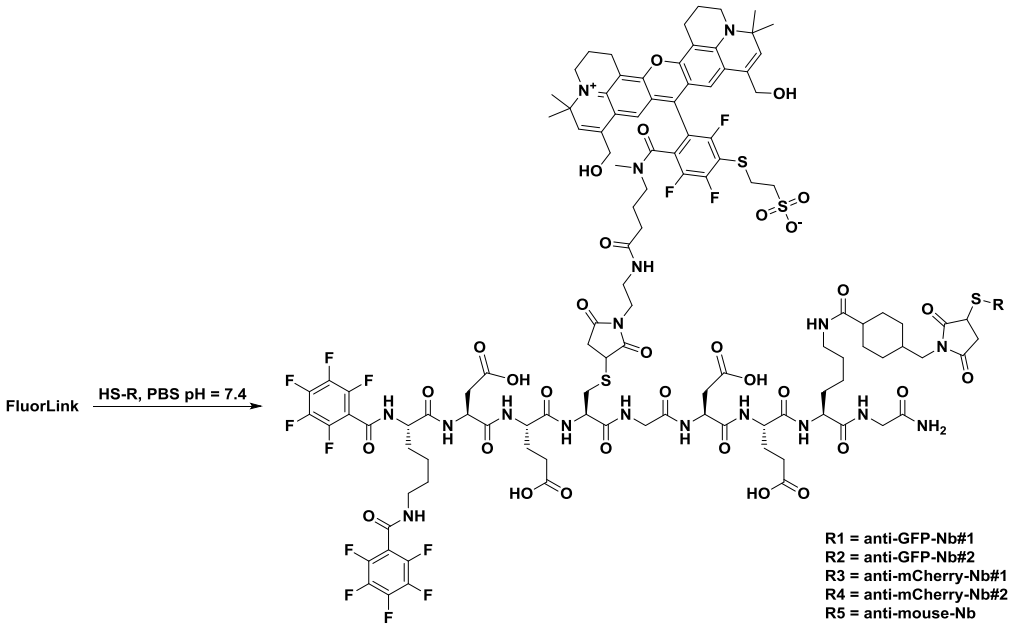
$C_{114}H_{127}F_{13}N_{18}O_{32}S_3$ [2604.52]

The peptide **2** (1.38 mg, 579 nmol, 1.0 eq) was dissolved first in a mixture of DIEA (1.51 µL, 8.69 µmol, 15.0 eq) in dry DMF (400 µL) and incubated for 5 min at RT under argon atmosphere. Then, succinimidyl 4-(*N*-maleimidomethyl)cyclohexane-1-carboxylate (SMCC) (1.10 mg, 3.29 µmol, 5.68 eq) in dry DMF (50 µL). After stirring for 5 h at RT and under light exclusion, the reaction mixture was diluted with 10% aq. acetonitrile and purified by HPLC. The product FluorLink (888 µg, 341 nmol, 59%)[2] was obtained as a blue solid after freeze-drying.

Analytical data: HPLC (C-18, analytical, gradient 40 → 85% B in 30 min): t_R = 11.60–13.59 min. *m/z* (ESI) = 905.6 [*M* + 5Na – 2H]$^{3+}$, 1335.9 [*M* + 3Na – H]$^{2+}$. *m/z* (HR-ESI-MS) = calculated: 890.5832 [*M* + 3Na]$^{3+}$, 905.2378 [*M* + 5Na – 2H]$^{3+}$, 1324.3801 [*M* + 2Na]$^{2+}$, 1335.3711 [*M* + 3Na – H]$^{2+}$, 1346.3621 [*M* + 4Na – 2H]$^{2+}$, found: 890.5838 [*M* + 3Na]$^{3+}$, 905.2374 [*M* + 5Na – 2H]$^{3+}$, 1324.3783 [*M* + 2Na]$^{2+}$, 1335.3719 [*M* + 3Na – H]$^{2+}$ 1346.3600 [*M* + 4Na – 2H]$^{2+}$.

[1] The use of the disulfide-reducing reagent TCEP in excess is not recommended. This leads to deactivation of the maleimide fluorophore (HPLC and ESI-MS confirmed, data not shown here). Furthermore, reduction prior coupling is not required here, due to the stability of peptide **2** against oxidation under applied and even long storage conditions.

[2] Yield can be further increased by adding 1% TFA to the dilution solution to neutralize DIEA. Otherwise hydrolyzed maleimide will be also found in the crude solution (HPLC and ESI-MS confirmed, data not shown here).



Supplementary Scheme S2: Synthesis of FluorLink-nanobodies.

General conjugation procedure for FluorLink-nanobody

The freeze-dried C,N-terminal thiol containing nanobody (1.0 eq) was resuspended in degassed milliQ-H_2O (400 µL/mg). Degassed tris(hydroxymethyl)aminomethane (Tris) buffer (22.2 µL, 1 mM, pH = 7.5) was added to the solution to get a final buffer concentration of 100 mM Tris-buffer. Tris(2-carboxyethyl)phosphine hydrochloride (TCEP) in degassed milliQ-H_2O (10 eq) was added to the nanobody solution and incubated for 30 min at ambient temperature. The buffer was changed to degassed PBS by passing through a column prepacked with Sephadex® G25. Afterwards, the resulting solution was cooled to 0 °C followed by the addition of FluorLink in anhydrous DMSO (4 eq per nanobody equal 2 eq per thiol). The reaction mixture was incubated for 2 h at 0 °C under argon atmosphere excluding light. Then, the reaction mixture was purified by size-exclusion (SE)-HPLC followed by analysis of the fractions by SDS-PAGE.

The FluorLink-nanobodies anti-fluorescent proteins (FP) were aliquoted and shock-frozen in liquid nitrogen, while the FluorLink-nanobody anti-mouse IgG was first concentrated, diluted with glycerol (1:1, PBS/glycerol) and then aliquoted. Afterwards, the blue-colored stock solutions were stored at –21 °C by excluding light and avoiding several freeze-thaw cycles.

4. Biochemical and Cellular Experiments

Experiments and sample preparations were adapted from previously published protocols.[1–4]

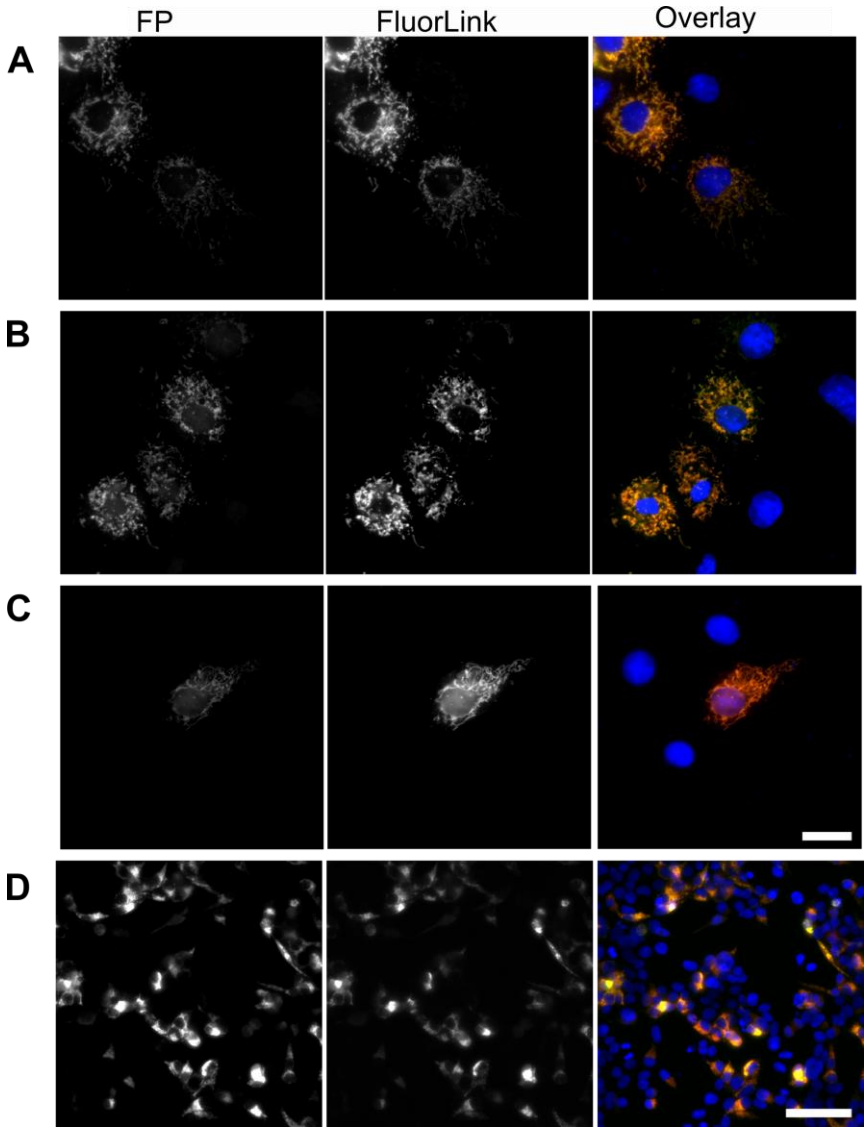
Cell preparation. Cells were cultured in complete DMEM medium containing 10% FCS, 4 mM L-glutamine and 100 U/mL penicillin and streptomycin. For the experiment, cells in DMEM without antibiotics were seeded into 12-well-plates containing PLL-coated coverslips and incubated at 37 °C in a humid atmosphere with 5% CO_2. For transfection the cells were treated as instructed by the manufacturer of Lipofectamine® 2000. Before immunostaining, cells were fixed with 4 % PFA in PBS for 30–40 min at RT, quenched with 100 mM glycine in PBS, washed briefly with PBS, and permeabilized and blocked with 2.5 % BSA and 0.1 % Triton X-100 in PBS for 15 min. [Following POIs were shown in here: TOM70-GFP (transfected) in figure 2A, S1A and S2A), TOM70-mCherry (transfected) in Fig. 2B and S1B, PMP70 (endogenous) in Fig. 3A, Lamin-B2 (endogenous) in Fig. 3B, TOM70-GFP (transfected) in Fig. S2A and α-Tubulin (endogenous) in Fig. S2B]. **Direct immunostaining approach targeting FP.** The fixed, blocked and permeabilized cells were incubated with both FluorLink-

nanobodies anti-FP (#1 and #2) for 1 h at RT in 1.25 % BSA and 0.05 % Triton X-100 in PBS and washed with with PBS for 3×5 min. The following FluorLink-nanobodies were used for staining: FluorLink-nanobody anti-GFP #1 and #2 (40–50 nM each) in Figure 2A and S1A and FluorLink-nanobody anti-mCherry #1 and #2 (40–50 nM each) in Fig. 2B and S1B). **Indirect immunostaining approach targeting POI.** The fixed, blocked and permeabilized cells were incubated with the primary antibody for 1 h at RT [anti-PMP70 (1:100), goat anti-rabbit lgG-Alexa488 (1:50), mouse anti-goat (1:50) for Fig. 3A, mouse anti-Lamin-B2 (1:100) forFig. 3B, mouse anti-GFP (1:100) for Fig. S2A, mouse anti-α-Tubulin (1:100) for Fig. S2B]. Afterwards the cells were washed with permeabilization/blocking solution (3×5 min) and incubated with FluorLink-nanobody anti-mouse lgG (25–50 nM) in 1:1-diluted blocking/permiabilization solution for 1 hour at RT followed by final wash with PBS (2×5 min). **Hoechst Labeling and Embedding for Epifluorescence Imaging Purpose.** The cell nuclei were labeled using 4 μM Hoechst in PBS for 5 minutes. This was followed by washing with PBS, high-salt PBS and PBS (5–10 min each) and mounting in Mowiol 4-88. **Plastic Embedding and Thin Sectioning.** The fixed and labeled cells were dehydrated with increasing amount of EtOH in ddH_2O, 30% EtOH (1×10 min), and 50 % EtOH (3×10 min). The samples were then incubated sequentially in a mixture of LR White and 50% EtOH (1:1) for 1 h, and in pure LR White for 1 h. Afterwards, the cells were covered with capsules (Beem Inc., West Chester, PA, USA) and embedded in LR White plus LR White accelerator (London Resin Company Ltd) for 30 min on a pre-cooled metal-plate followed by 90 min incubation at 60 °C. The embedded samples were finally cut, using an EM UC6 ultramicrotome (Leica Microsystems, Wetzlar, Germany), into sections at a thickness of 200 nm which were subsequently placed on silicon wafers (Siegert Wafer GmbH, Aachen).

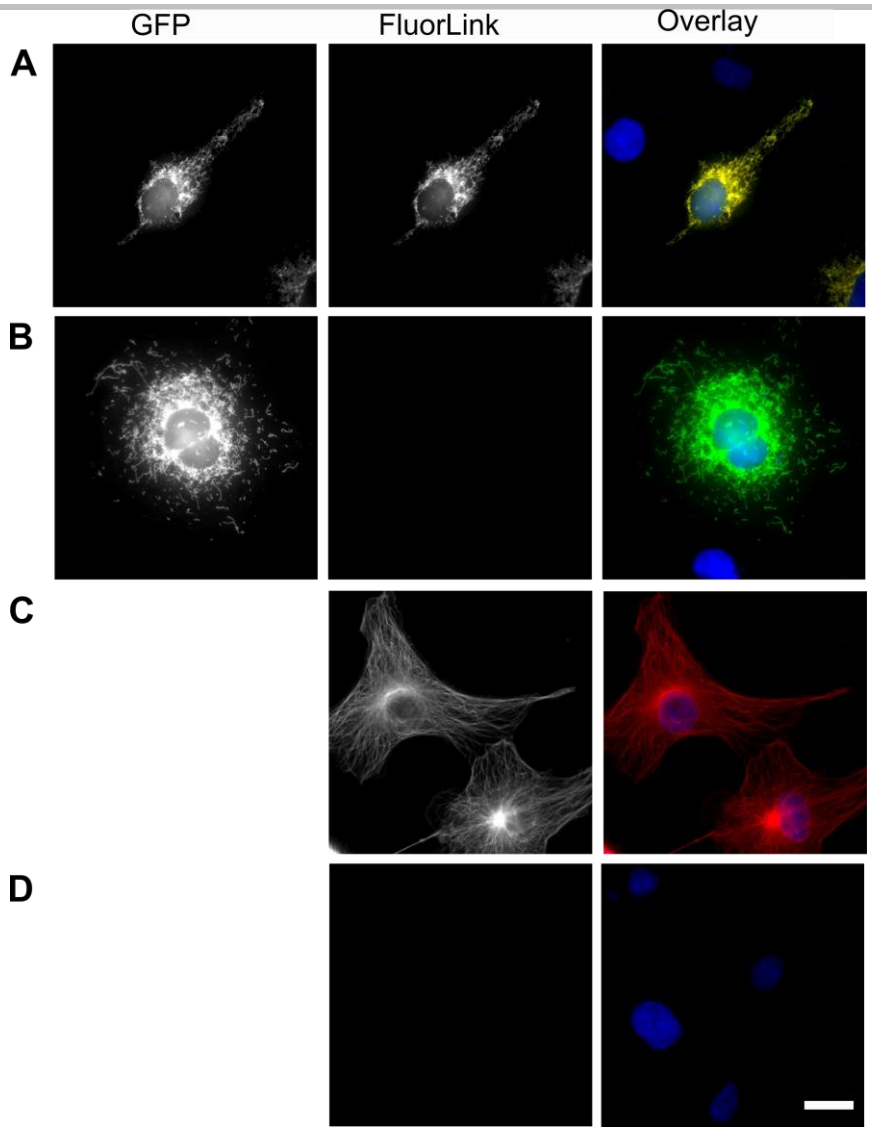
5. Imaging

Epifluorescence Microscopy. Proof-of-principle images (shown in Supporting Figure S1 and S2) were taken with an inverted microscope Nikon Ti Eclipse equipped with a 0.75 NA/100× (oil) or 40×(air) objective and Nikon DS-Qi2 camera (Nikon GmbH, Düsseldorf, Germany). To aid identification of probe-positive cells in nanoSIMS, overview images of embedded slices (shown in Figure 2) were taken with a plan apochromat 20× 0.75 NA air objective, an HBO-100W Lamp, and an IXON X3897 Andor (Belfast, Northern Ireland, UK) camera controlled via the NIS-Elements AR software (version 4.20; Nikon). **Secondary Ion Mass Spectrometry (SIMS) Measurement and Data Analysis.** NanoSIMS measurement was performed using a nanoSIMS 50L (Cameca, France) equipped with an 8 kV Cesium primary ion source. The primary current of ~2.5 pA was used to erode the sample surface producing secondary ions which were then focused through the ion optics and separated into different mass per charge (m/z) by a magnetic sector. The secondary ions then reached parallel electron multipliers for detection. To obtain the steady state of ionization before imaging, the sample was implanted at the primary current ~110 pA. The images were acquired with the raster size 12x12 μm to 20x20 μm with 256x256 pixels, or 37 μm with 512x512 pixels. The ions of interest are $^{19}F^-$, $^{12}C^{14}N^-$ which will be simply presented as ^{19}F, and $^{12}C^{14}N$ in the rest of the paper**. Data Analysis.** Processing of the NanoSIMS data, including image exportation, image stacking, line profile and ratio measurements were performed using ImageJ with the plugin OpenMIMS.

6. Supplementary Figures

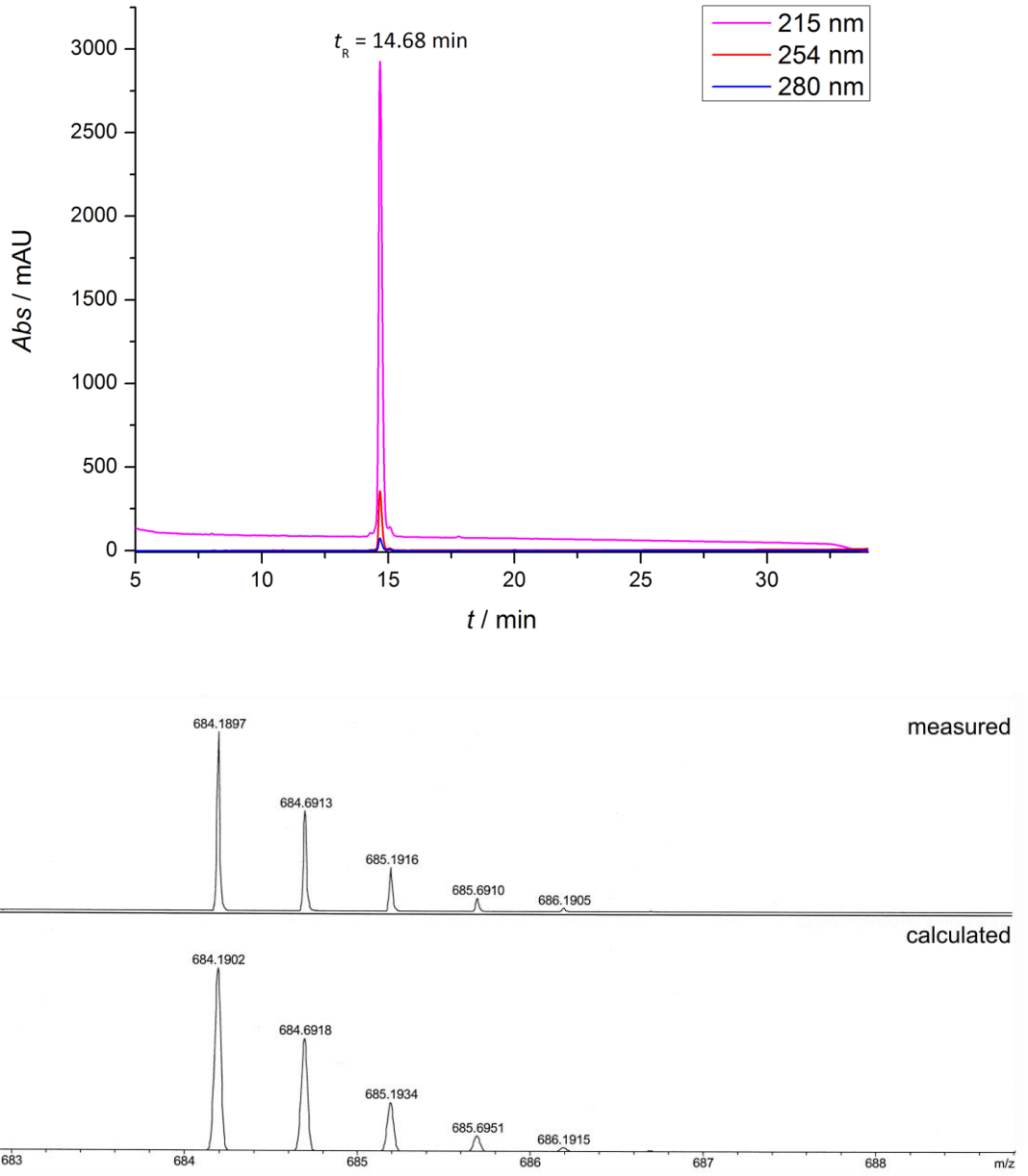


Supplementary Figure S1. General observations from epifluorescence micrographs of COS-7 (A to C) and HEK293 (D) cells immunostained with FluorLink anti-FP for the direct approach. A) TOM70-GFP is specifically labeled with FluorLink-nanobody anti-GFP #1. Colocalization of the FluorLink (red) and the GFP (green) labeling in the overlay (Hoechst is shown in blue). B) FluorLink-nanobody anti-GFP #2 shows high affinity for TOM70-GFP, too. C) Labeling with both FluorLink-nanobodies anti-GFP #1 and #2 is confirmed, as expected, while non-transfected cells remained unstained. The fluorescence signal was brighter when both nanobodies were used by keeping the same microscope settings. D) The specific immunostaining of TOM70-mCherry was observed with the next set of nanobodies, FluorLink-nanobody anti-mCherry #1 and #2. Scale bar: 20 µm.

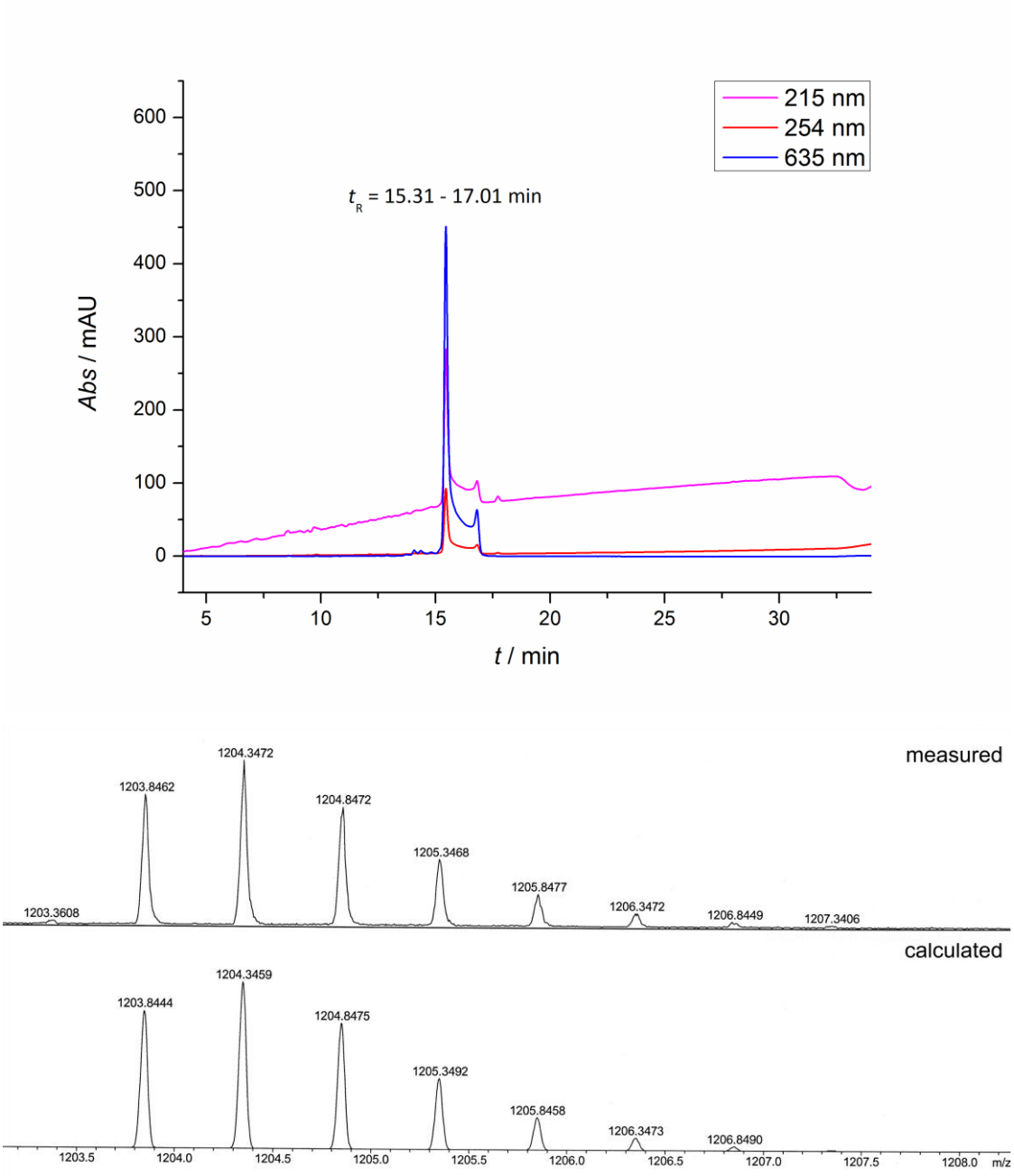


Supplementary Figure S2. General observations from epifluorescence micrographs of COS-7 cells immunostained with FluorLink anti-mouse IgG for the indirect approach. A) TOM70-GFP is specifically labeled with FluorLink-nanobody anti-mouse IgG when stained with primary mouse antibody anti-GFP before. Colocalization of the FluorLink (red) and the GFP (green) labeling in the overlay (Hoechst is shown in blue). B) In the absence of primary atibody, no FluorLink-signal is observed, as expected. C) Specific labeling of α-Tubulin with primary mouse antibody anti-α-Tubulin and FluorLink anti-mouse IgG is confirmed by the specific protein structure and from D) the negative control, when no primary mouse antibody was used. Scale bar: 20 µm.

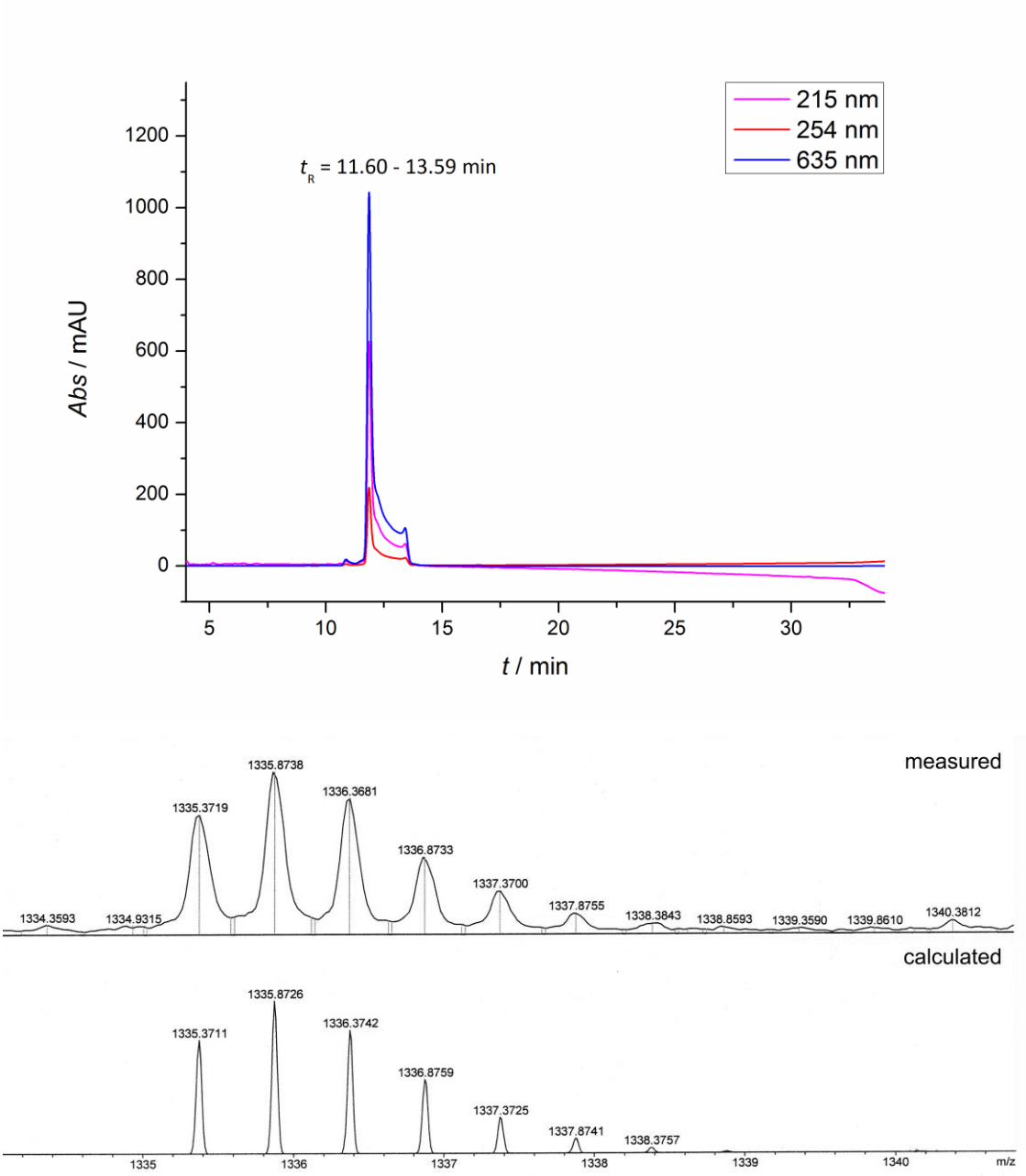
7. Supplementary Spectra



Supplementary Spectra S1: HPLC-chromatogram of compound **2** (top) and HR-ESI-MS-spectra of compound **2**, $[M + 2H]^{2+}$ selected as example (bottom).



Supplementary Spectra S2: HPLC-chromatogram of compound **3** (top) and HR-ESI-MS-spectra of compound **3**, $[M + Na + H]^{2+}$ selected as example (bottom). The broad product peak in the HPLC chromatogram is specific for the fluorophore Star635 under applied conditions.



Supplementary Spectra S3: HPLC-chromatogram of FluorLink (top) and HR-ESI-MS-spectra of FluorLink, $[M + 3Na - H]^{2+}$ selected as example (bottom). The broad product peak in the HPLC chromatogram is specific for the fluorophore Star635 under applied conditions.

8. References

[1] S. Kabatas, P. Agüi-Gonzalez, K.-A. Saal, S. Jähne, F. Opazo, S. O. Rizzoli, N. T. N. Phan, *Angew. Chem. Int. Ed.*, 2019, **58**, 3438–3443; *Angew. Chem.*, 2019, **131**, 3476–3481.

[2] I. C. Vreja, S. Kabatas, S. K. Saka, K. Kröhnert, C. Höschen, F. Opazo, U. Diederichsen, S. O. Rizzoli, *Angew. Chem. Int. Ed.*, 2015, **54**, 5784–5788; *Angew. Chem.*, 2015, **127**, 5876–5880.

[3] S. Kabatas, I. C. Vreja, S. K. Saka, C. Höschen, K. Kröhnert, F. Opazo, S. O. Rizzoli, U. Diederichsen, *Chem. Commun.,* 2015, **51**, 13221–13224.

[4] S. K. Saka, A. Vogts, K. Kröhnert, F. Hillion, S. O. Rizzoli and J. T. Wessels, *Nat. Commun.,* 2014, **5**, 3664.