Electronic Supplementary Information

An iodine-containing probe as a tool for molecular detection in secondary ion mass spectrometry

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1. Materials and General Methods for Chemical Synthesis

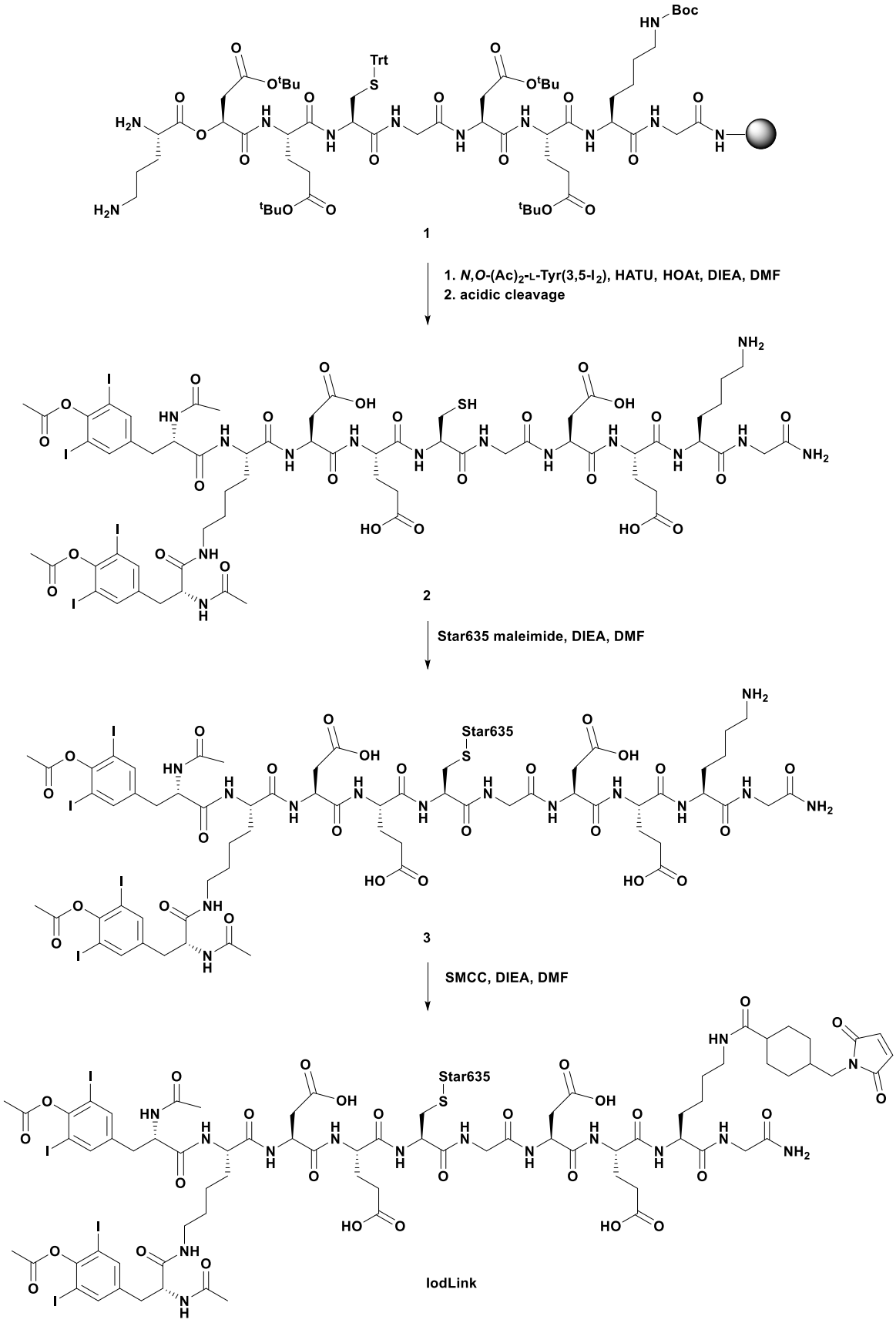
Reagents. Starting materials and reagents were of the highest grade available from commercial sources and were used as delivered. Fmoc-protected L-amino acids, coupling reagents and Sieber amide resin were purchased from Iris Biotech (Marktredwitz, Germany) and Carl Roth (Karlsruhe, Germany). Star635 maleimide was obtained from Abberior (Göttingen, Germany). Non-conjugated nanobody FluoTag®-X2 anti-mouse Immunoglobin κIg (N1202) was 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 the 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. Matrix-assisted laser desorption/ionization (MALDI) spectra were recorded on a MALDI TOF Autoflex Speed 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 (trifluoroacetic acid, solvent A) and acetonitrile/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-labelled 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 and Opti-MEM were obtained from Thermo Fisher Scientific (Darmstadt, Germany). The rabbit polyclonal anti-PMP70 (ab85550) was delivered 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 anti-α-Tubulin (302 211) was supplied from Synaptic Systems (Göttingen, Germany). FluorLink-nanobodies-anti-green fluorescent protein (GFP) #1 and #2 were prepared as described in our previous publication.[1] **Cells.** A cell line derived from monkey kidney (COS-7) was used in this study. The cells were maintained in culture in Dulbecco's Modified Eagle Medium (DMEM, Thermo Fisher Scientific, Darmstadt, Germany) with 5% fetal bovine serum (FBS), 2 mM L-glutamine, 60 U/mL penicillin, and 60 U/mL streptomycin**. Constructs.** The plasmid TOM70-eGFP was produced in-house and validated via sanger sequencing.

3. Synthesis of IodLink-nanobody



Scheme S1: Synthesis of IodLink.

_N_,_O_-L-Tyr[3,5-I_2]-Lys(_N_,_O_-L-Tyr[3,5-I_2])-Asp-Glu-Cys-Gly-Asp-Glu-Lys-Gly-NH_2 (2)

$C_{63}H_{84}I_4N_{14}O_{25}S$ [1977.12]

The first step shown in Supplementary Scheme S1 includes the coupling of *N*,*O*-diacetyl-3,5-diiodo-L-tyrosine to H-Lys-Asp(O^{t}Bu)-Glu(O^{t}Bu)-Cys(Trt)-Gly-Asp(O^{t}Bu)-Glu(O^{t}Bu)-Lys(Boc)-Gly-Sieber amide resin (**1**). The synthetic procedure for peptide **1** was adapted from previous publications.[1–3] The peptide **1** on resin (10.0 µmol, 1.0 eq) was swollen for 1 h at room temperature (RT) in *N*,*N*-dimethylformamide (DMF). *N*,*O*-diacetyl-3,5-diiodo-L-tyrosine (25.8 mg, 50.0 µmol, 5.0 eq), *O*-(7-azabenzotriazol-1-yl)-*N*,*N*,*N*',*N*'-tetramethyluronium hexafluorophosphate (HATU, 17.2 mg, 45.0 µmol, 4.5 eq) and 1-hydroxy-7-azabenzotriazole (HOAt, 6.80 mg, 50.0 µmol, 5.0 eq) were dissolved in DMF (400 µL). The solution was activated with *N*,*N*-diisopropylethylamine (DIEA, 17.4 µL, 100 µmol, 10.0 eq) and subsequently added to the resin. The coupling was carried out by microwave irradiation (10 min, 50 °C, 25 W). After washing the resin with DMF (3×), dichloromethane (DCM, 3×) and DMF (3×), double coupling was performed followed by final washing with DMF, DCM, NMP, DCM, MeOH, DCM (3× each) and drying under vacuum. Cleavage of the peptide from the Sieber amide resin and simultaneous deprotection of protecting groups was obtained by using a 10% TFA/H_2O/EDT/TIS cleavage cocktail (94:2.5:2.5:1, *v*/*v*/*v*/*v*) in DCM (3 × 20 min). The collected fractions were mixed with concentrated cleavage cocktail (1:1) and incubated for an additional hour. The resulting solution was concentrated under an argon stream followed by precipitation using cold Et_2O. The resulting suspension was centrifuged at –4 °C. The supernatant was discarded and the compound was washed with Et_2O (2×) and dried in *vacuo*. The crude peptide was purified by reverse-phase HPLC. The iodinated compound **2** was obtained as a white solid.

Analytical data: HPLC (C-18, analytical, gradient 20 → 80% B in 30 min): t_R = 16.73 min. *m/z* (ESI) = 989.1 $[M + 2H]^{2+}$, 1000.1 $[M + H + Na]^{2+}$, 1977.2 $[M + H]^+$, 1999.2 $[M + Na]^+$, 987.1 $[M - 2H]^{2-}$, 1975.2 $[M - H]^-$. *m/z* (HR-ESI-MS) = calculated: 989.0888 $[M + 2H]^{2+}$, 1011.0708 $[M + 2Na]^{2+}$, 987.0743 $[M - 2H]^{2-}$, found: 989.0886 $[M + 2H]^{2+}$, 1011.0698 $[M + 2Na]^{2+}$, 987.0747 $[M - 2H]^{2-}$.

_N_,_O_-L-Tyr[3,5-I_2]-Lys(_N_,_O_-L-Tyr[3,5-I_2])-Asp-Glu-Cys(Star635)-Gly-Asp-Glu-Lys-Gly-NH_2 (3)

$C_{114}H_{138}I_4N_{19}O_{35}S_3$ [2995.25]

Under argon atmosphere, the peptide **2** (1.94 mg, 982 nmol, 2.0 eq) was suspended in degassed PBS (pH 7.4, 50 mM sodium phosphate buffer + 100 mM NaCl, 500 µL).[1] Abberior® Star635-maleimide in dry DMF (50.0 µL, 500 µg, 491 nmol, 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 afterwards overnight at +4 °C. The crude product was purified by HPLC. The compound **3** (1.49 mg, 497 nmol, 99%) was obtained as a blue solid after freeze-drying.

Analytical data: HPLC (C-18, analytical, gradient 20 → 80% B in 30 min): t_R = 19.45–20.77 min. *m/z* (ESI) = 1013.8 $[M + H + 2Na]^{3+}$, 1509.2 $[M + H + Na]^{2+}$. *m/z* (HR-ESI-MS) = calculated: 1013.4918 $[M + H + 2Na]^{3+}$, 1508.7430 $[M + H + Na]^{2+}$, 1519.7340 $[M + 2Na]^{2+}$, found: 1013.4905 $[M + H + 2Na]^{3+}$, 1508.7418 $[M + H + Na]^{2+}$, 1519.7323 $[M + 2Na]^{2+}$.

_N_,_O_-L-Tyr[3,5-I_2]-Lys(_N_,_O_-L-Tyr[3,5-I_2])-Asp-Glu-Cys(Star635)-Gly-Asp-Glu-Lys(MCC)-Gly-NH_2, IodLink

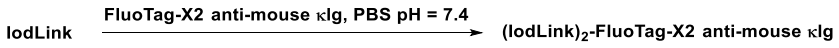
$C_{126}H_{151}F_3I_4N_{20}O_{38}S_3$ [3214.49]

The peptide **3** (1.50 mg, 500 nmol, 1.0 eq) was dissolved first in a mixture of DIEA (1.31 µL, 7.50 µmol, 15.0 eq) in dry DMF (333 µL) and incubated for 5 min at RT under argon atmosphere. Then, succinimidyl 4-(*N*-maleimidomethyl)cyclohexane-1-carboxylate (SMCC, 1.67 mg,

[1] The use of the disulfide-reducing reagent TCEP in excess is not recommended. This leads to the 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.

5.00 µmol, 10.0 eq) in dry DMF (167 µL). After stirring for 5 h at RT and under light exclusion, the reaction mixture was diluted with 10% aq. acetonitrile/1% TFA[2] and purified by HPLC. The product **IodLink** (1.17 mg, 364 nmol, 73%) was obtained as a blue solid after freeze-drying.

Analytical data: HPLC (C-18, analytical, gradient 30 → 80% B in 30 min): t_R = 18.48–20.12 min. *m/z* (ESI) = 1094.2 $[M + 3Na]^{3+}$, 1629.8 $[M + 2Na]^{2+}$, 1070.2 $[M - 3H]^{3-}$, 1605.8 $[M - 2H]^{2-}$. *m/z* (HR-ESI-MS) = calculated: 1093.8489 $[M + 3Na]^{3+}$, 1629.2788 $[M + 2Na]^{2+}$, 1069.8524 $[M - 3H]^{3-}$, 1605.2823 $[M - 2H]^{2-}$, found: 1093.8483 $[M + 3Na]^{3+}$, 1629.2773 $[M + 2Na]^{2+}$, 1069.8567 $[M - 3H]^{3-}$, 1605.2871 $[M - 2H]^{2-}$.



Scheme S2: Synthesis of IodLink-nanobody anti-mouse κIg.

General procedure for the conjugation of IodLink-nanobody

The procedure was adapted from previous publication.[2] 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 = 8) 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 (100 mM, 10.0 eq) was added to the nanobody solution and incubated for 20 min at 300 rpm and RT. The buffer was changed to degassed PBS (pH = 7.4) by passing through a column prepacked with Sephadex® G25. Afterwards, the resulting solution was cooled to 0 °C followed by the addition of IodLink in anhydrous DMSO (4.0 eq per nanobody equal 2.0 eq per thiol). The reaction mixture was incubated for 2 h at 0 °C under an 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 IodLink-nanobody was first concentrated and then diluted with glycerol (1:1, PBS/glycerol). After determination of the concentration, stock solutions were prepared and stored at –21 °C by excluding light and avoiding several freeze-thaw cycles.

4. Biochemical and Cellular Experiments

Cell culturing and transfection

COS-7 cells were cultured in a humidified incubator at 37 °C and 5% CO2, plated on poly-L-lysine (PLL) coated glass coverslips, incubated in Dulbecco's MEM with the addition of 10% FBS, 4 mM L-glutamine and 0.6% penicillin and streptomycin. The cells to be immunostained with FluorLink-nanobodies-anti-GFP #1 and #2 were transfected with a plasmid containing TOM70 fused to eGFP. This was done by using 2.5% lipofectamine 2000® and 300 ng of plasmid in Optimem medium incubated for 16 h in the incubator. The cells were then fixed as described below.

Immunostaining

Anti-tubulin immunostaining for IodLink-nanobody anti-mouse κIg concentration test. COS-7 cells were fixed with pre-cooled methanol for 20 min at -20 °C. The cells were briefly washed with PBS. To prevent unspecific bindings, the cells were then incubated in a blocking solution of 3% bovine serum albumin (BSA) in PBS for 30 min at RT. For the primary staining, cells were incubated with the mouse

[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).

monoclonal anti-α-Tubulin (302 211, Synaptic Systems, Göttingen, Germany) using a concentration of 1:500 diluted in PBS-BSA for 1 h at RT. Then the cells were washed three times for 5 min with PBS and incubated for 1 h with IodLink-nanobody anti-mouse κlg (concentrations of 20–40 nM diluted in PBS-BSA) followed by 3 washes with PBS for 5 min.

PMP70 and GFP immunostainings with IodLink-nanobody anti-mouse κlg and FluorLink-nanobodies anti-GFP. COS-7cells, which were previously transfected with a plasmid containing TOM70 fused to eGFP, were fixed for 30 min with a solution of 4% paraformaldehyde (PFA) in PBS at RT. To quench the reactive aldehydes, the samples were incubated in a solution of 0.1 M glycine in PBS for 30 min at RT. To prevent unspecific binding and allow the penetration of the immunoprobes, the cells were then incubated in a blocking and permeabilizing solution containing 3% BSA and 0.1% Triton X-100 in PBS for 30 min at RT. The cells were then incubated in blocking solution (1.5 % BSA + 0.05 % Triton X-100 in PBS) with rabbit polyclonal anti-PMP70 (1:100) for 1 h at RT. Then the cells were washed three times for 5 min with blocking solution and incubated in blocking solutions with the secondary goat anti-rabbit antibody (#111-545-144, Dianova, Hamburg, Germany) (1:50) for 1 h at RT. Subsequentially, cells were incubated in blocking solutions with the mouse anti-goat antibody (#205-005-108, Dianova, Hamburg, Germany) (1:50) for 1 hour at RT. Afterwards, the cells were washed three times for 5 min with blocking solution. Last, cells were incubated with the anti-mouse IodLink nanobody (20 nM) and anti-GFP FluorLink nanobodies #1 and #2 (45 nM) for 2 h at RT.

Cells were post-fixed by incubating them in a solution of 4% PFA + 2% glutaraldehyde in PBS for 1 h at RT and then the fixative was quenched for 30 min in a solution of 0.1 M glycine in PBS.

Negative control samples. To test the unspecific binding of IodLink-nanobody anti-mouse κlg in NanoSIMS experiments, all negative control cells presented in this work were processed in the exact same manner than their positive counterparts, carrying out the incubations with the respective primary antibodies and IodLink-nanobody anti-mouse κlg, but excluding the intermediate antibodies.

Resin embedding and sectioning

The different batches of fixed and stained cells were embedded in LR White embedding medium (medium grade, London Resin Company, London, UK), following a slightly modified version of previously published protocols. [4-6] The cells were partially dehydrated by applying increasing concentrations of ethanol; first incubating them in a solution of double-distilled water (ddH2O) and 30% ethanol for 10 min, afterward for 10 min in a solution of ddH2O and 50% ethanol and three times for 10 min in a solution of ddH2O and 70% ethanol. All steps were performed at RT with the samples rotating at 50 rpm on a plate shaker.

Afterward, the samples were incubated for 1 h in a mixture of 70% ethanol (in ddH2O) and LR White (1:1). The coverslips were then transferred onto a new plate containing pure LR White shaking at 50 rpm for 1 h at RT. After the incubation, the coverslips were dried and placed onto a pre-cooled metal plate. Beem® plastic capsules (BEEM Inc., West Chester, PA, USA) were placed on top of the areas of interest of the coverslips. 10 mL of LR White were premixed with one drop of LR White accelerator (London Resin Company, London, UK) for 30 s and added to the bottom of the Beem® capsules. After an initial hardening phase of 30 min, the Beem® capsules were completely filled up with a fresh mixture of LR White and LR White accelerator.

Samples were then polymerized at 60 °C for 90 min. After cooling, coverslips were removed from the samples, which were then cut into 100-200 nm thin sections using an ultramicrotome (EM UC6, Leica Microsystems, Wetzlar, Germany). The slices were placed onto Silicon wafers (Siegert Wafer GmbH, Aachen, Germany) for NanoSIMS imaging.

5. Imaging

Fluorescence Imaging

Fluorescent imaging was performed with a Nikon inverted epifluorescence microscope (Nikon Corporation, Chiyoda, Tokyo, Japan), equipped with a 60× Plan Apochromat oil immersion objective (NA 1.4) an HBO 100 W lamp and an IXON X3897 (Andor Camera, Belfast, UK). Images were obtained using the acquisition software NiS-Elements AR (Nikon Corporation, Chiyoda, Tokyo, Japan).

NanoSIMS Imaging

NanoSIMS imaging was performed using a NanoSIMS 50L (CAMECA, Gennevilliers, France) equipped with the 8 kV Cs^+ primary ion source.

Samples containing only IodLink-nanobody anti-mouse κlg. The detectors were set to collect $^{12}C^{14}N^-$, $^{32}S^-$, and $^{127}I^-$ ions. $^{127}I^-$ appeared as a single peak, with no isobaric interferences observed at mass 127. The mass resolving power (MRP) was adjusted to ensure the discrimination between the isobaric interferences present at mass 26 and 32, by using an entrance slit of 30×180 µm and an aperture slit of 350×250 µm, to obtain an MRP value of ~3700 for $^{12}C^{14}N^-$ and ~1700 for $^{32}S^-$.
To reach a stable yield of secondary ions, slightly bigger areas than the ones that were finally imaged, were implanted by applying a primary ion current of ~30 pA for 1 min (primary aperture D1:1), resulting in a primary ion dose of ~ $1x10^{15}$ ion/cm2. Subsequently, the areas of interest, ranging from 18x18 to 50x50µm, were analysed by applying a primary ion current of ~4.5 pA (primary aperture D1:2) and a dwell time of 5.7ms/pixel to obtain 5 layers ion images of 256x256pixels.

Samples containing IodLink-nanobody anti-mouse κlg and FluorLink-nanobody anti-GFP. The detectors were set to $^{19}F^-$, $^{12}C^{14}N^-$, and $^{127}I^-$. No isobaric interferences were observed at mass 127. The mass resolving power of the instrument was adjusted to ensure the discrimination between possible isobaric interferences on mass 19 and 26 by using an entrance slit of 20×140 µm and an aperture slit of 150×150 µm, to obtain the MRP value of ~1500 and ~7600 for $^{19}F^-$ and $^{12}C^{14}N^-$, respectively.
To reach the steady-state of the secondary ion yield, slightly bigger areas than the ones that were finally imaged, were first implanted with a primary ion current of ~70 pA (primary aperture D1:1) resulting in a primary ion dose of ~$2x10^{15}$, and subsequently analysed applying a primary ion current of ~5 pA (primary aperture D1:3) on the areas of interest with a dwell time of 5.07 ms/pixel. Areas of interest ranging from 20×20 µm to 60×60 µm were imaged to obtain 5 consecutive ion images of 256x256 pixels.

Data Analysis

NanoSIMS images were exported using the Open MIMS Image plugin[3] from ImageJ (NIH, Bethesda, USA). Drift correction and the addition of 5 consecutive layers were also carried out by this software. Self-written Matlab scripts (the Mathworks Inc, Natick, MA) were used for further analysis of the data.
To compare the signal intensity obtained from the IodLink immunostainings, we manually selected circular ROIs of 5 pixels in diameter on areas that showed the highest abundance of ^{127}I in all samples, including the labelled (also referred as positive samples) and the negative control samples. The labelled samples were immunostained following the entire immunostaining protocol (n=35), while the negative control samples were immunostained with the primary antibody and the IodLink nanobody, but did not include the intermediate antibodies that link the IodLink nanobodies to the primary one (n=35). To determine the difference in the signal intensity of ^{127}I in these two sample groups, a Kolmogorov-Smirnov test was employed. To test the correlation between the signal intensities of ^{19}F and ^{127}I provided by the FluorLink and the IodLink labelling, we analysed the Pearson's correlation coefficient between the respective NanoSIMS images (n=18), and compared it to the correlation coefficient obtained for the $^{12}C^{14}N$ and ^{32}S images (n=8).

[3] The plugin has been developed at the National Resource for Imaging Mass Spectrometry, now Bringham and Women´s Hospital Center for NanoImaging (http://nano.bwh.harvard.edu).

6. Supplementary Figures

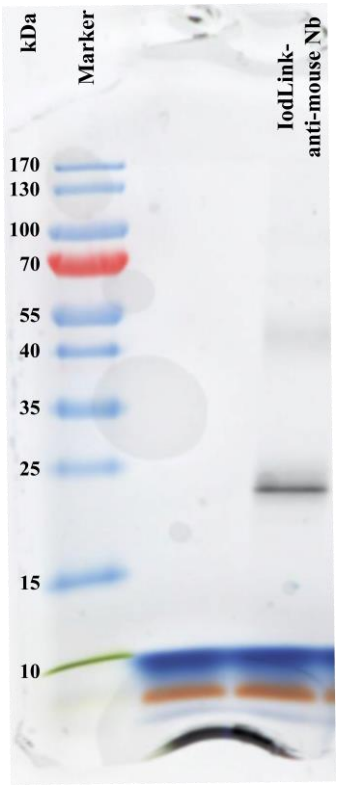


Figure S1. SDS PAGE of IodLink-nanobody anti-mouse κlg after SE-HPLC purification. The expected mass range of ~22 kDa is indicated by the SDS gel. FI-Red: Fluorescence, Epi-RGB (red), light 630 nm, filter Cy5. Marker = PageRuler protein ladder (ThermoScientific, Germany).

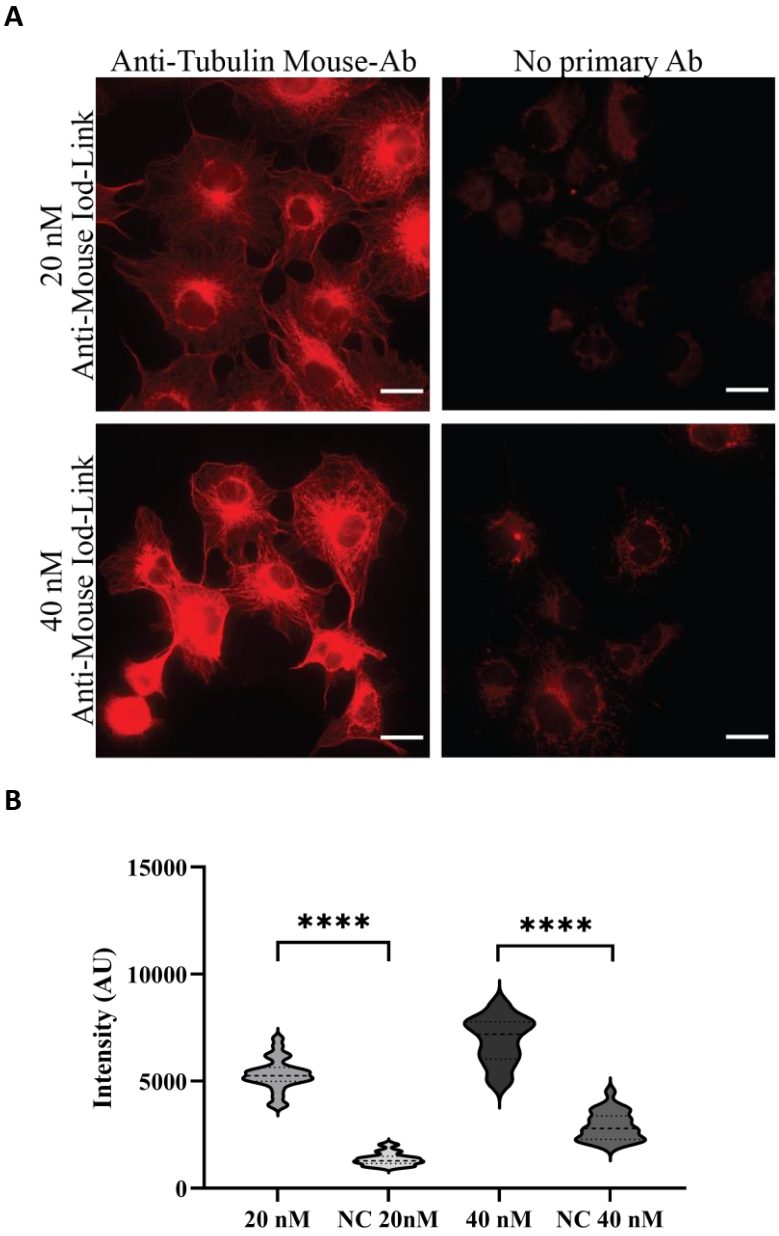


Figure S2. Optimization of IodLink-nanobody concentration with fluorescence imaging. COS-7 cells immunostained with IodLink-nanobody anti-mouse κlg. (**A**) Left column: mouse monoclonal anti-α-Tubulin + IodLink-nanobody anti-mouse κlg (upper panel 20 nM and lower panel 40 nM). Right column: negative control; no primary antibody + IodLink-nanobody anti-mouse κlg (upper panel 20 nM and lower panel 40 nM). Scale bars: 25 µm. (**B**) Plot of Kolmogorov-Smirnov test to compare the fluorescence levels between the higher intensity areas on positive and negative control cells. The positive samples were stained with the entire sequence of immunoprobes incubating them respectively with 20 or 40 nM of IodLink-nanobody anti-mouse κlg, while the control samples (NC) were incubated with respectively 20 or 40 nM of IodLink-nanobody anti-mouse κlg, but excluding the primary immunoprobes. On each condition, the middle dash line represents the median, while the upper and lower dash lines indicate the 25th and 75th percentiles, respectively. The mean intensity values were obtained from highly labelled areas, selecting them on 5 cells per image and 5 images per condition (n=25 per condition); *** = p<0.0001.

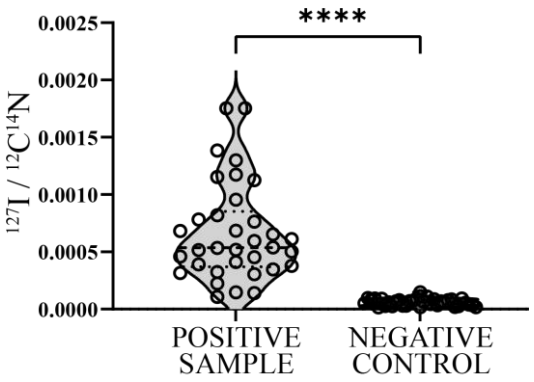


Figure S3. PMP70 immunostaining with IodLink-nanobody anti-mouse κlg. Plot of the $^{127}I^{-}/^{12}C^{14}N^{-}$ ratio values to compare the areas of highest iodine presence between the positive samples, immunostained following the entire sequence of probes (n=35 circular ROIs in 7 cells), and the negative control cells, whose immunostaining did not include the intermediate antibodies (n = 35 ROIs in 7 cells); The difference was clearly significant (p<0.001) as determined by a Kolmogorov-Smirnov test: ****=p<0.001. For each condition, the middle dash line represents the median, while the upper and lower dash lines indicate the 25th and 75th percentiles, respectively.

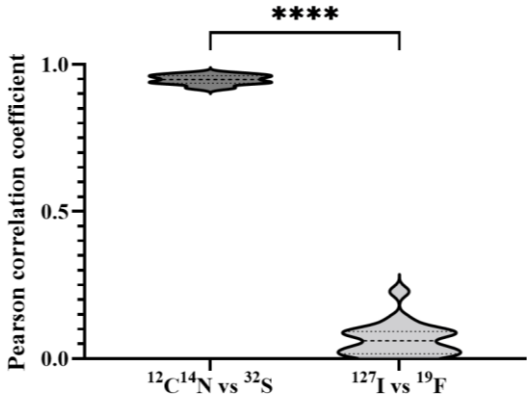
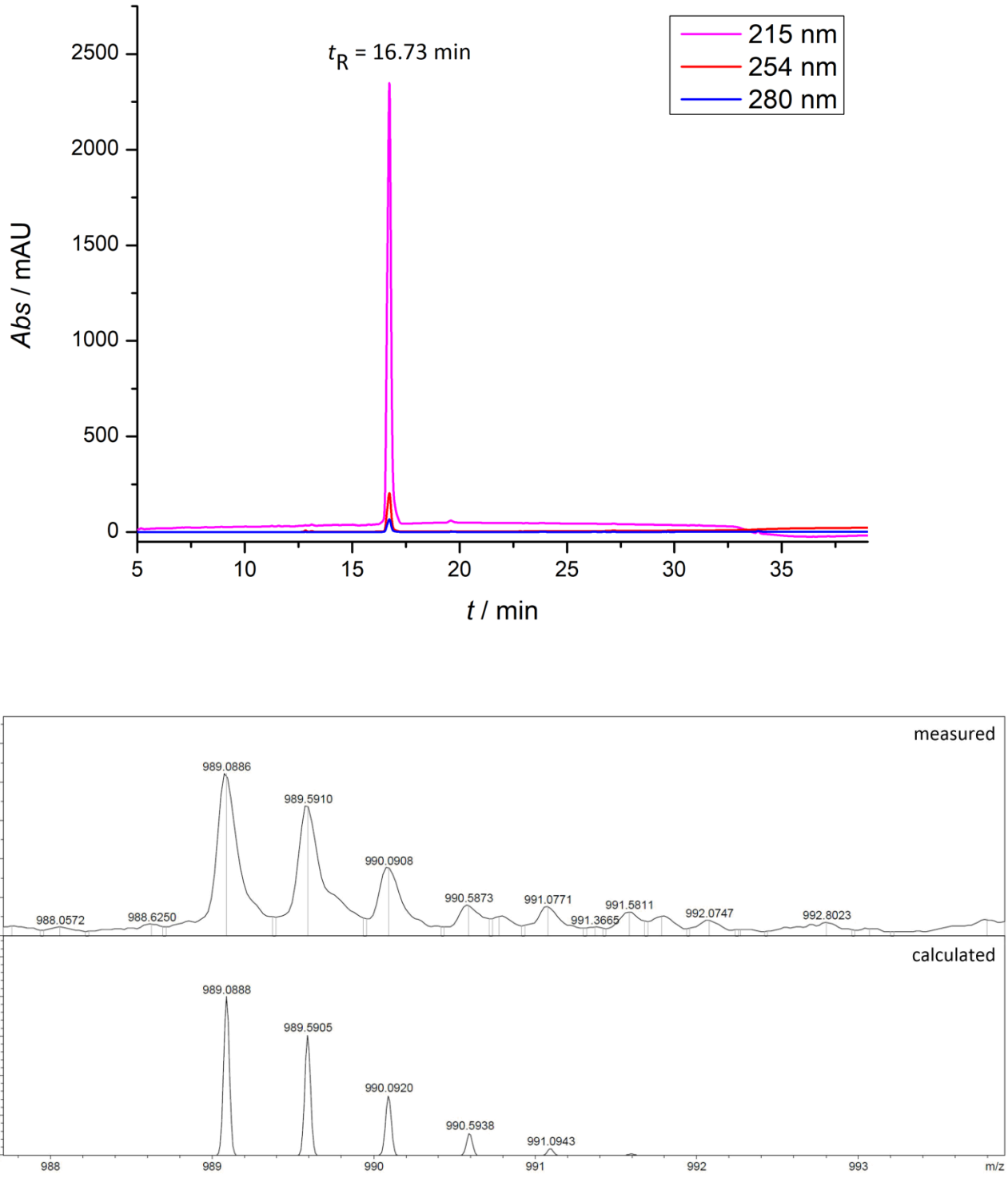
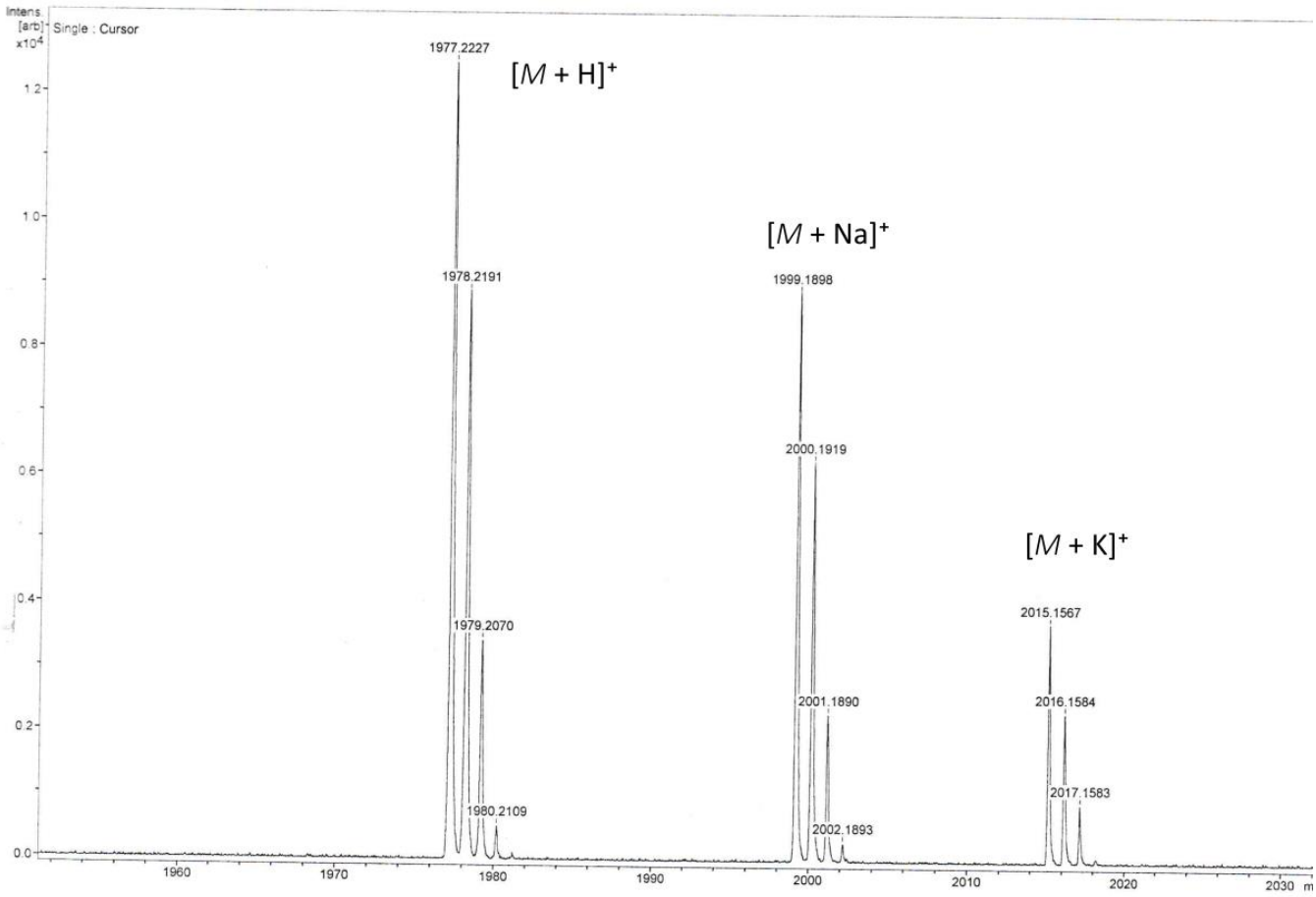


Figure S3. Analysis of the dual-isotope immunostaining using FluorLink- and IodLink-nanobodies. The Pearson's correlation coefficient was determined on the cells immunostained with both probes (FluorLink- and IodLink-nanobodies), to analyse the correlation between $^{12}C^{14}N$ and ^{32}S, or ^{127}I and ^{19}F, respectively. The correlation of the latter is very low, indicating that the two labels reveal different organelles, as expected. The difference is highly significant (P =0.0000712, Mann-Whitney rank sum test). ^{127}I vs ^{19}F (n=18); $^{12}C^{14}N$ vs ^{32}S (n=8). The middle dash line represents the median, while the upper and lower dash lines indicate the 25th and 75th percentiles, respectively.

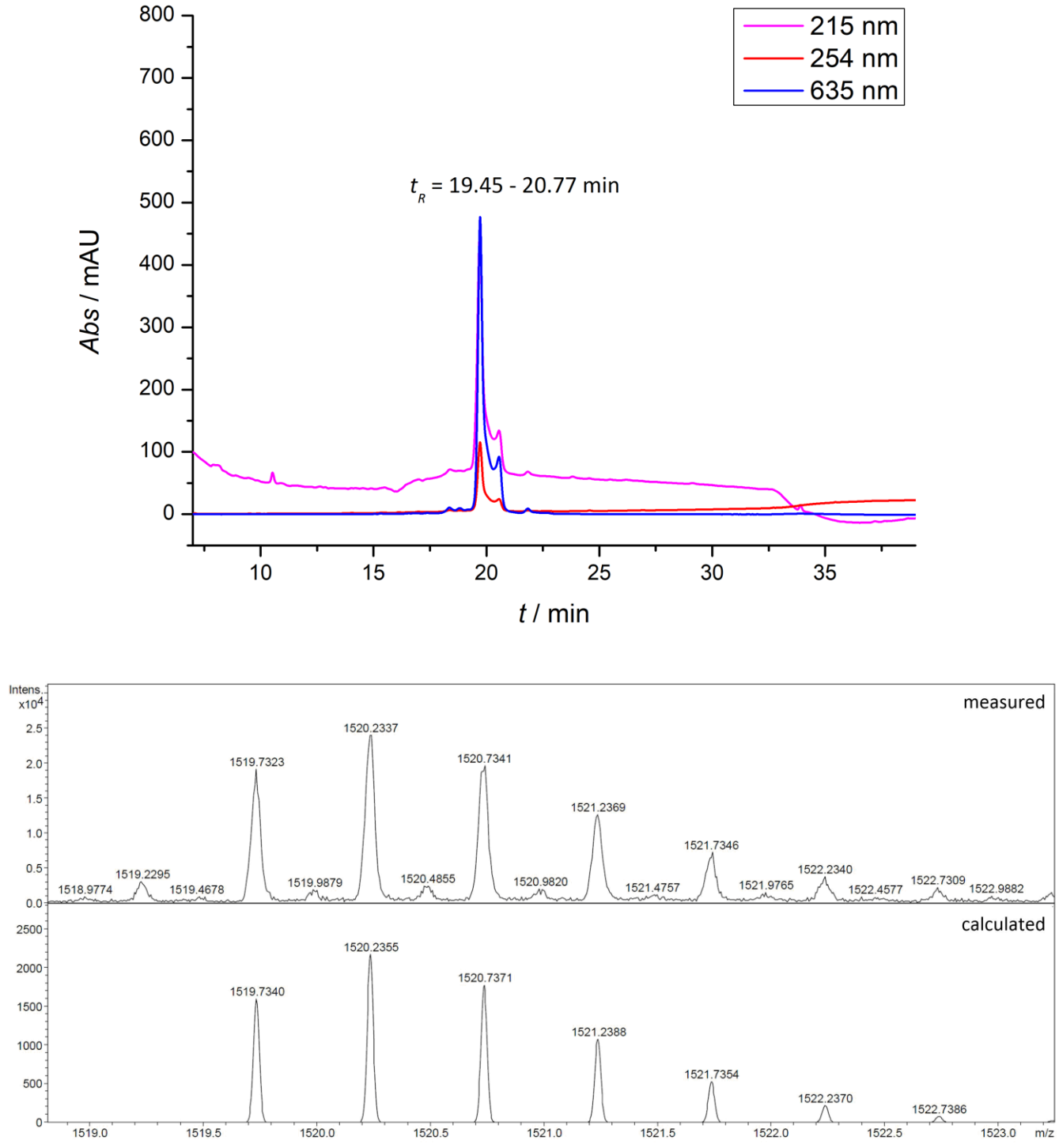
7. Supplementary Spectra



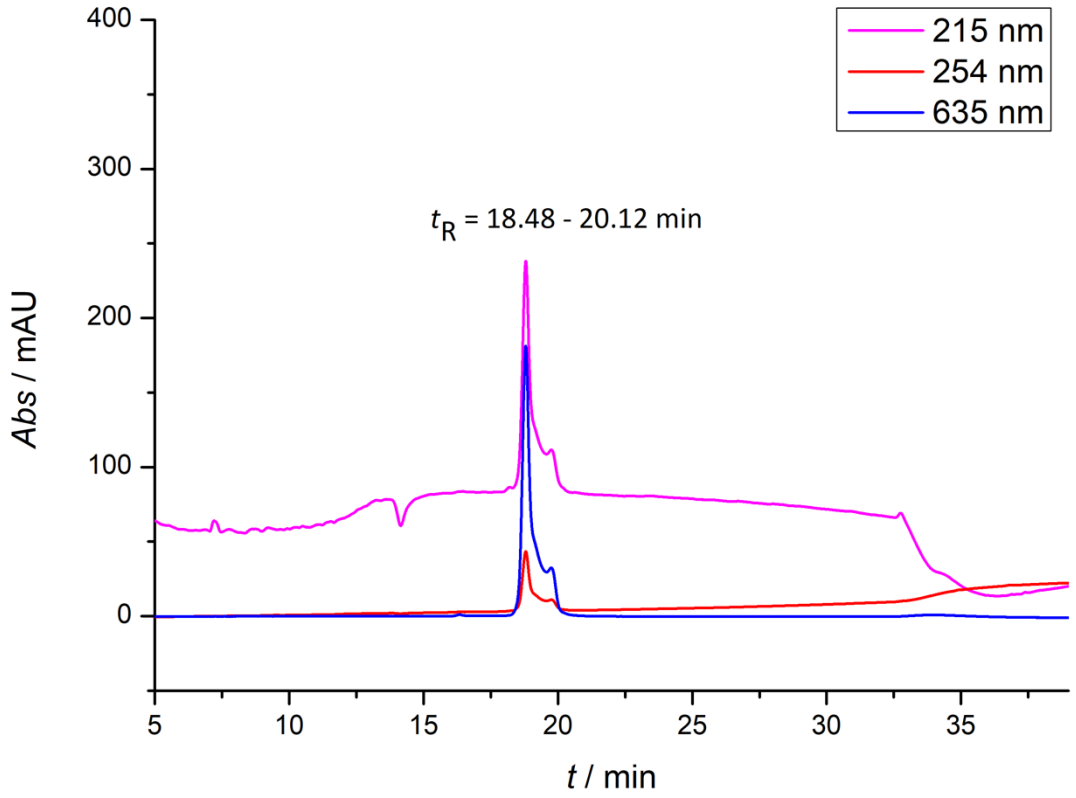
Spectra S1: HPLC-chromatogram of compound **2** (top) and HR-ESI-MS-spectra of compound **2**, $[M + 2H]^{2+}$ selected as example (bottom).

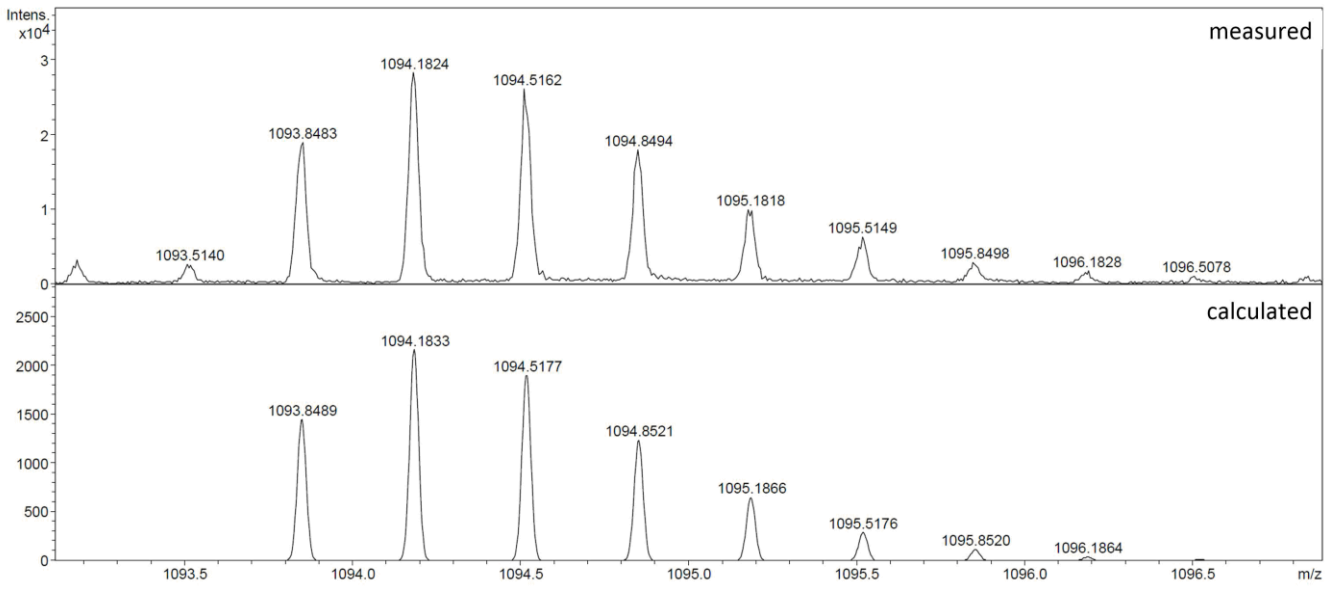


Spectra S2: MALDI-ToF mass spectrum of compound **2** obtained in reflectron positive mode with α-cyano-4-hydroxycinnamic acid (CHA) as the matrix and a laser power up to 55%.



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





Spectra S4: HPLC-chromatogram of IodLink (top) and HR-ESI-MS-spectra of IodLink, $[M + 3Na]^{3+}$ selected as example (bottom). The broad product peak in the HPLC chromatogram is specific for the fluorophore Star635 under applied conditions.

8. References

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Author Contributions

S.K.G and S.O.R. conceptualized the project. S.K.G. performed all chemistry work. S.S.I performed the immunolabelling experiments and fluorescence imaging. F.O provided materials and supervised nanobody conjugation and their applications in IF. S.J. performed the plastic embedding. P.A.G. performed the SIMS imaging and N.T.N.P. supervised all SIMS work. P.A.G. and S.O.R. analysed the data. S.K.G., P.A.G and S.O.R. wrote the initial draft.