

## Supporting Information

### Hybrid imaging labels; providing the link between mass spectrometry-based molecular pathology and theranostics

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#### Material and methods

##### *General*

All chemicals were obtained from commercial sources and used without further purification. The reactions were monitored by thin layer chromatography (TLC). High performance liquid chromatography (HPLC) was performed on a Waters HPLC system using a 1525EF pump and a 2489 UV detector. For preparative HPLC a Dr. Maisch GmbH Reprosil-Pur 120 C18-AQ 10  $\mu\text{m}$  (250  $\times$  20 mm) column was used (12 mL/min). For semi-preparative HPLC a Dr. Maisch GmbH Reprosil-Pur C18-AQ 10  $\mu\text{m}$  (250  $\times$  10 mm) column was used (5 mL/min). For analytical HPLC a Dr. Maisch GmbH Reprosil-Pur C18-AQ 5  $\mu\text{m}$  (250  $\times$  4.6 mm) column was used and a gradient of 0.1% TFA in  $\text{H}_2\text{O}/\text{CH}_3\text{CN}$  95:5 to 0.1% TFA in  $\text{H}_2\text{O}/\text{CH}_3\text{CN}$  5:95 in 20 minutes (1 mL/min) was employed. Molecular mass spectrometry was performed on a Bruker microflex MALDI-TOF. NMR spectra were taken using a Bruker DPX-300 spectrometer (300 MHz  $^1\text{H}$  NMR, 75 MHz  $^{13}\text{C}$  NMR) and the chemical shifts are given in ppm ( $\delta$ ) relative

to tetramethylsilane (TMS). Abbreviations used include singlet (s), doublet (d), doublet of doublets (dd), triplet (t) and unresolved multiplet (m).

#### *Boc-Lys(Cbz)-Abu-OBzl*

Boc-Lys(Cbz)-OH (7.61 g, 20 mmol) and benzyl 4-aminobutanoate p-tosylate (H- $\gamma$ -Abu-OBzl · p-tosylate, 7.31 g, 20 mmol) were dissolved in CH<sub>3</sub>CN (250 mL) and DiPEA (3.40 mL, 20 mmol) was added. The solution was cooled to 0 °C and DCC (4.33 g, 21 mmol) was added. The mixture was stirred over the weekend at RT. The resulting suspension was filtered and the filtrate was concentrated. EtOAc was added to the residue and the organic phase was washed with 5% NaHCO<sub>3</sub> (3×), 5% citric acid (3×) and brine, dried with Na<sub>2</sub>SO<sub>4</sub>, filtered and concentrated. The product was purified with column chromatography (gradient of EtOAc/hexane 1:1 to EtOAc/hexane 2:1) yielding 5.8 g (10.4 mmol, 52%) of a yellowish oil that slowly solidified. MS: [M+Na]<sup>+</sup> calculated 578.3, found 578.0; [M+K]<sup>+</sup> calculated 594.4, found 594.0. <sup>1</sup>H NMR (CDCl<sub>3</sub>, 300 MHz)  $\delta$  = 1.35 (m, 2H, CH<sub>2</sub>), 1.42 (s, 9H, tBu), 1.48 (m, 2H, CH<sub>2</sub>), 1.56 (m, 2H, CH<sub>2</sub>), 1.81 (m, 2H, CH<sub>2</sub>), 2.39 (t, 2H, CH<sub>2</sub>), 3.17 (dd, 2H, CH<sub>2</sub>), 3.27 (dd, 2H, CH<sub>2</sub>) 4.00 (m, 1H, CH), 4.97 (m, 1H, NH), 5.08, 5.10 (2 s, 4H, 2 CH<sub>2</sub> benzyl), 5.19 (m, 1H, NH), 6.49 (m, 1H, NH), 7.34 (m, 10H, Ar). <sup>13</sup>C NMR (CDCl<sub>3</sub>, 75 MHz)  $\delta$  = 22.6, 24.7, 29.6, 31.7, 32.0, 38.9, 40.5 (7 CH<sub>2</sub>), 28.4 (3 CH<sub>3</sub>), 54.5 (C $\alpha$ ) 66.5, 66.7 (2 CH<sub>2</sub> benzyl), 128.2, 128.4, 128.6, 128.7 (10 CH Ar), 135.9, 136.7 (2 C Ar), 156.7 (CO Boc and Cbz), 172.3, 173.2 (2 CO).

#### *DTPA(tBu)-Lys(Cbz)-Abu-OBzl*

Boc-Lys(Cbz)-Abu-OBzl (2.78 g, 5 mmol) was dissolved in CH<sub>2</sub>Cl<sub>2</sub> (20 mL). H<sub>2</sub>O (0.2 mL) and TFA (20 mL) were added and the mixture was stirred for 1 h at RT. The volatiles were evaporated and CH<sub>2</sub>Cl<sub>2</sub> and an aqueous solution of 5% NaHCO<sub>3</sub> was added. The layers were separated and the aqueous layer was further extracted with CH<sub>2</sub>Cl<sub>2</sub>. The combined organic layers were dried with Na<sub>2</sub>SO<sub>4</sub>, filtered and concentrated. The obtained oil was dissolved in CH<sub>2</sub>Cl<sub>2</sub> (25 mL).

DTPA(tBu)<sub>4</sub> (3.09 g, 5 mmol) and PyBOP (2.60 g, 5 mmol) were dissolved in CH<sub>2</sub>Cl<sub>2</sub> (25 mL) and DiPEA (2.18 mL, 12.5 mmol) was added. This solution was added to the solution containing the intermediate. The reaction mixture was stirred for over the weekend at RT and then washed with an aqueous solution of 5% NaHCO<sub>3</sub> and subsequently brine, dried with Na<sub>2</sub>SO<sub>4</sub>, filtered and concentrated. The product was purified with column chromatography (first EtOAc/hexane 1:4 with 0.5% TEA, then EtOAc/hexane 1:2 with 0.5% TEA, then EtOAc with 0.5% TEA, then EtOAc/MeOH 1:9 with 0.5% TEA) yielding 4.6 g (4.36 mmol, 87%) of a yellow oil. MS: [M+H]<sup>+</sup> calculated 1055.3, found 1055.9. <sup>1</sup>H NMR (CDCl<sub>3</sub>, 300 MHz) δ = 1.43-1.56 (m, 40H, 2 CH<sub>2</sub>, 12 CH<sub>3</sub>), 1.77-1.91 (m, 4H, 2 CH<sub>2</sub>), 2.37 (t, 2H, CH<sub>2</sub>), 2.58-2.64 (m, 4H, 2 CH<sub>2</sub> DTPA), 2.74-2.77 (m, 4H, 2 CH<sub>2</sub> DTPA), 3.14-3.31 (m, 6H, CH<sub>2</sub> DTPA, 2 CH<sub>2</sub>), 3.36 (s, 8H, 4 CH<sub>2</sub> DTPA), 4.25-4.35 (m, 1H, CH), 5.08, 5.10 (2 s, 4H, 2 CH<sub>2</sub> benzyl), 5.20, 6.86, 8.32 (3 m, 3H, 3 NH), 7.29-7.35 (m, 10H, Ar).

#### *DTPA(tBu)-Lys-Abu*

DTPA(tBu)-Lys(Cbz)-Abu-OBzl (3.5 g, 3.32 mmol) was dissolved in MeOH (50 mL). Ammonium formate (4.19 g, 66.4 mmol) and 10% Pd/C (66 mg) were added and the mixture was stirred overnight at RT. The suspension was filtered over Celite and

concentrated. The residue was dissolved in EtOAc and washed with an aqueous solution of 5% NaHCO<sub>3</sub>, dried with Na<sub>2</sub>SO<sub>4</sub>, filtered and concentrated. The product was purified with column chromatography (gradient of CH<sub>2</sub>Cl<sub>2</sub>/MeOH 4:1 with 0.5% 30-33% NH<sub>3</sub> in H<sub>2</sub>O to MeOH with 0.5% 30-33% NH<sub>3</sub> in H<sub>2</sub>O) yielding 2.17 g (2.61 mmol, 79%) of an off-white foam. MS: [M+H]<sup>+</sup> calculated 831.1, found 831.2.

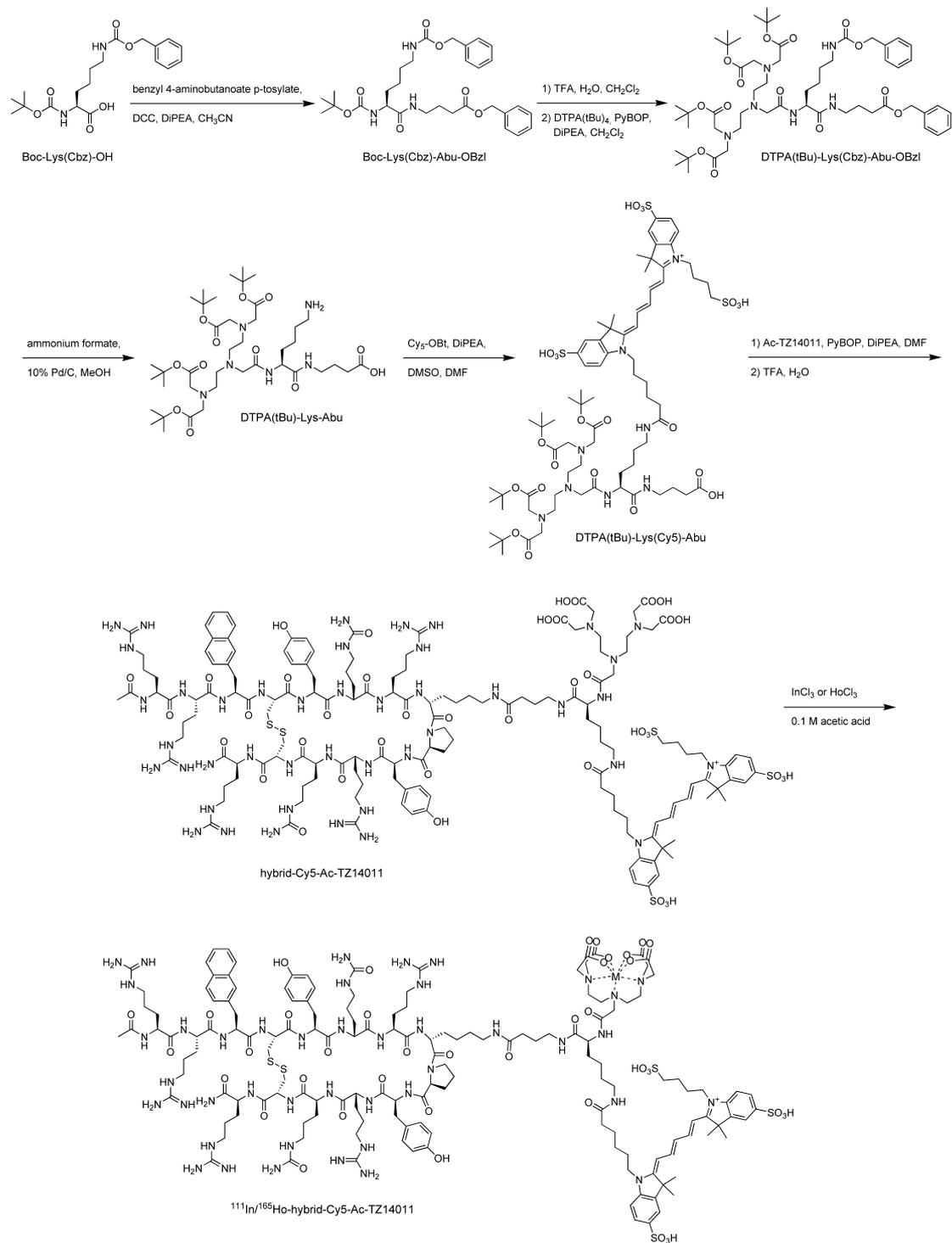
#### *DTPA(tBu)-Lys(Cy5)-Abu*

Cy5 (77 mg, 100 μmol), PyBOP (52 mg, 100 μmol) and HOBt (15 mg, 100 μmol) were dissolved in DMF (2 mL). DiPEA (87 μL, 500 μmol) was added and the solution was stirred for 1 h at RT. DTPA(tBu)-Lys-Abu was dissolved in DMSO/DMF 1:1 (2 mL) and DiPEA (35 μL, 500 μmol) was added. This solution was added to the activated Cy5 and the resulting mixture was stirred overnight at RT. The first purification step was column chromatography (gradient of CH<sub>2</sub>Cl<sub>2</sub>/MeOH 2:1 with 1% 30-33% NH<sub>3</sub> in H<sub>2</sub>O to CH<sub>2</sub>Cl<sub>2</sub>/MeOH 1:1 with 1% 30-33% NH<sub>3</sub> in H<sub>2</sub>O). The final purification was preparative HPLC using a gradient of 0.1% TFA in H<sub>2</sub>O/CH<sub>3</sub>CN 9:1 to 0.1% TFA in H<sub>2</sub>O/CH<sub>3</sub>CN 1:9 in 100 minutes (4 runs). The product was obtained as a blue fluffy solid (32.5 mg, 20.6 μmol, 21%) after pooling of the appropriate fractions and lyophilization. MS: [M]<sup>+</sup> calculated 1579.0, found 1578.4; [M+Na-H]<sup>+</sup> calculated 1601.0, found 1601.6.

#### *Hybrid-Cy5-Ac-TZ14011*

Ac-TZ14011 was synthesized according to described procedures.[1] For synthesis of hybrid-Cy5-Ac-TZ14011 Ac-TZ14011 (4.0 mg, 1.4 μmol), DTPA(tBu)-Lys(Cy5)-Abu (2.9 mg, 1.4 μmol) and PyBOP (0.7 mg, 1.4 μmol) were dissolved in DMF (1 mL) and DiPEA (3.7 μL, 21.5 μmol) was added. The solution was stirred overnight at RT and

the reaction mixture was concentrated. H<sub>2</sub>O (100 μL) and TFA (1.9 mL) were added and the solution was stirred for 3 h at RT. TFA was evaporated and the product was purified with semi-preparative HPLC using a gradient of 0.1% TFA in H<sub>2</sub>O/CH<sub>3</sub>CN 95:5 to 0.1% TFA in H<sub>2</sub>O/CH<sub>3</sub>CN 5:95 in 100 minutes. The product was obtained as a blue fluffy solid (2.0 mg) after pooling of the appropriate fractions and lyophilization. MS: [M]<sup>+</sup> calculated 3444.0, found 3445.6; [M+Na-H]<sup>+</sup> calculated 3466.0, found 3467.5; [M+H]<sup>2+</sup> calculated 1722.5, found 1723.3.



**Scheme S11. Synthesis and labeling of hybrid-Cy5-Ac-TZ14011.**

### Radiolabeling

Hybrid-Cy5-Ac-TZ14011 (50  $\mu\text{g}/100 \mu\text{L}$ ) was dissolved in aqueous acetic acid (0.1 M).

$^{111}\text{InCl}_3$  (10 MBq/50  $\mu\text{g}$  of compound,  $3.5 \cdot 10^{11}$   $^{111}\text{In}$  atoms; Covidien-Mallinckrodt)

was added and was allowed to incubate 1 hr. These preparations have a radiochemical purity of at least 95% after analysis by instant thin layer chromatography (ITLC) on 1x5 cm ITLC-SG paper strips (Agilent Technologies, USA) with phosphate buffered saline (PBS) as mobile phase. In all cases, labeling efficacy was >99%. In case of post-radiolabelling functionalization with  $^{165}\text{Ho}$ ,  $\text{HoCl}_3$  (1 equiv., 11  $\mu\text{L}$  from a 1 mM stock of  $\text{HoCl}_3$  in water, Sigma-Aldrich, Zwijndrecht) was added to the mixture and allowed to incubate for 1 hr. Before injection of ( $^{111}\text{In}$ )hybrid-Cy5-ActZ14011, the solution was diluted (1:1) with saline (0.90% w/v of NaCl in water).

#### *Holmium ( $^{165}\text{Ho}$ ) labeling*

Hybrid-Cy5-ActZ14011 was dissolved in aqueous acetic acid (0.1 M) at a concentration of 25  $\mu\text{M}$  as determined by UV/VIS spectroscopy. One equivalent of holmium in the form of  $^{165}\text{HoCl}_3$  (2.5  $\mu\text{L}$  from a 1 mM stock of  $\text{HoCl}_3$  in water;  $7.5 \cdot 10^{15}$   $^{165}\text{Ho}$  atoms) was added to the peptide solution and the mixture was incubated for 1 hr at room temperature (RT) to allow for  $^{165}\text{Ho}$  loading of the DTPA, resulting in ( $^{165}\text{Ho}$ )hybrid-Cy5-ActZ14011. This stock solution was diluted in culture medium or 0.90% w/v of NaCl in water, and respectively used in *in vitro* or *in vivo* experiments.

#### *Cell lines*

Human breast cancer cell lines with different levels of CXCR4 expression were cultured in Gibco's minimum essential medium (MEM) enriched with 10% fetal bovine serum and penicillin/streptomycin (all Life Technologies Inc.). Cells were kept under standard culturing conditions. High CXCR4 expressing MDAMB213 X4 cells

were kindly provided by Dr. Luker (Center for Molecular Imaging, University of Michigan, Ann Arbor, US). In these cells CXCR4 expression was acquired after transfection with a GFP-tagged version of the human CXCR4-gene, which resulted in the presence of green fluorescent CXCR4 on the cell membrane and in the lysosomes of the cells (Figure SI 1). Parental MDAMB231 cells with basal (low) expression of CXCR4 were used as a negative control.

#### *Determination of Dissociation Constants*

For results obtained with both flow- and mass cytometry the normalized geometric means were fitted with equations in the GraphPad Prism 5 software. The  $K_D$  values of the hybrid-Cy5-Ac-TZ14011 (measured with both flow cytometry and mass cytometry) was calculated using the “Binding – Saturation, One site – Total” nonlinear regression equation:  $y = \frac{B_{max} \cdot X}{K_D + X} + NS \cdot X + Background$

#### *Sample preparation for imaging of cells*

MDAMB231 and MDAMB231 X4 cells were seeded on glass bottom culture dishes (for confocal imaging; MatTek corporation) or 12 mm glass slides (for mass spectrometry imaging; STARFROST) and were placed in the incubator overnight.

#### *In vivo tumor model*

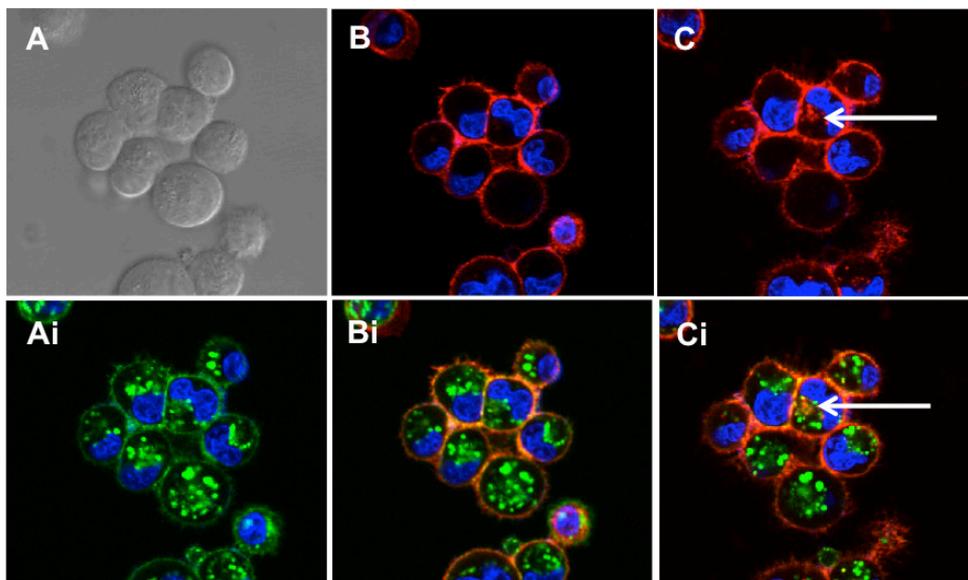
Balb/c nude mice (Harlan; 6-8 weeks of age) were anesthetized using a Hypnorm (VetaPharma Ltd.)/Dormicum (Midazolam; Roche)/ water solution (1:1:2; 5  $\mu$ L/g ip). Via a small incision, the inguinal lymph node was excised, where after  $2.0 \times 10^6$

MDAMB231 X4 cells were injected into the remaining tissue of the fourth mammary gland. Pain relief was given via intraperitoneal injection of 10  $\mu$ L Temgesic (Buprenorphine, 5mg/ml, Actavis) in 1mL 0.90% w/v of NaCl in water, at 30 min prior to and 24 h after the procedure. Approximately 8 weeks after transplantation, lesions were deemed suitable for further experiments (0.8–1 cm in diameter). All animal experiments were performed in accordance with the Dutch welfare regulations and were approved by the local ethics committee.

## Results

### *Evaluation of the location of binding and internalization*

When incubated at 4<sup>o</sup>C, hybrid-Cy5-Ac-TZ14011 was shown to bind to the CXCR4 present on the membrane of MDAMB231 X4 cells, while no staining was seen within the CXCR4 containing lysosomes (Figure SI1 A and B). Longitudinal imaging at RT revealed internalization of hybrid-Cy5-Ac-TZ14011 within 15 minutes (Figure SI 1C and Ci; white arrow)



**Figure S1. Membranous staining and internalization of hybrid-Cy5-Ac-TZ14011 over time.**

Fluorescence confocal imaging showing A) a white light image of the MDAMB231 X4 cells. Ai) unstained MDAMB231 X4 cells with intrinsic GFP-tagged CXCR4 present on the membrane and in lysosomes (in green). B) Membranous staining of the cells after incubation hybrid-Cy5-Ac-TZ14011 at 4°C (peptide in red). Bi) Overlay of the intrinsic GFP signal (in green) and stained receptors (in red; co-localization depicted in orange). C) Internalization of hybrid-Cy5-Ac-TZ14011 (white arrow for example) into the cytoplasm via lysosomes after incubation of the cells at 37°C. Ci) Overlay of intrinsic- (in green) and peptide-based signal (in red) is depicted in orange. In all fluorescent images: nucleus stained in blue.

**References**

1. Hanaoka H, Mukai T, Tamamura H, Mori T, Ishino S, Ogawa K, et al. Development of a <sup>111</sup>In-labeled peptide derivative targeting a chemokine receptor, CXCR4, for imaging tumors. *Nucl Med Biol.* 2006; 33: 489-94.