Labeling of Antibody Formats

For FMT/CT, antibody formats were labeled with Alexa750 (SAIVI™ Rapid Antibody Labeling Kit). The compounds were transferred to the reaction vials containing the lyophilized Alexa Fluor® dye and incubated for one hour in the dark (protein:dye conjugation ratio for mAb=1:5, for $F(ab')_2$=1:4 and Fab=1:3). During the labeling process the reactive N-hydroxysuccinimide (NHS) ester group of the fluorochrome reacts with the amines of the compound molecule to yield stable amide bonds. After the conjugation, the fluorescent conjugate was purified by size-exclusion chromatography. The protein:Alexa750dye ratio for mAb was 1:3.46, for $F(ab')_2$ was 1:2.56 and for Fab was 1:2.44.

For PET/MRI, antibody formats were conjugated with a chelator via NHS ester chemistry. Bifunctional chelator NODAGA-NHS (Chematech, Dijon, France) was added to the antibody at a molar ratio of 55:1 and incubated at 4°C for 24 h. Excess of chelator was removed by repeated ultrafiltration with 0.25 M sodium acetate (pH 6). The constructs were radiolabeled with ^{64}Cu as described previously [14] with the same molar excess for all formats, typically resulting in 3-4 chelator moieties per protein. The ^{64}Cu solution was buffered to pH 6 using 0.5 M ammonium acetate and the NODAGA-conjugated antibody was added at a ratio of 2 µg/MBq. After incubation for 1 h at 42°C, the incorporation of the ^{64}Cu was analyzed by thin layer chromatography (Agilent iTLC-SG, 0.5 M sodium citrate pH 5) and high performance size exclusion chromatography (Phenomenex BioSep-SEC-s3000, saline sodium citrate) [14].

Tissue Homogenization

Specimens were homogenized and 100 mg of each sample was dissolved in 1 mL lysis buffer (10 mM Tris, 137 mM NaCl, 1% Triton, 10% Glycin supplemented with protease inhibitor).

ECLIA Analysis

ECLIA was performed to quantify the antibody amounts in tissue homogenates and blood. A volume of 65 µL of a biotin-labeled capture antibody (IgG specific for kappa light chain) and 65 µL of ruthenium-labeled detection antibody were incubated with 10 µL of the samples for 4.5 minutes. Next, 60 µL streptavidin-coated magnetic beads binding to biotin were added to capture the antibody complex. Chemiluminescent emission signal was induced by a voltage in the measuring cell of the Elecsys® (Cobas, E411, Roche, Penzberg, Germany) leading to the oxidation of tripropylamine and ruthenium label. The ECL signal was detected by a photomultiplier at 620 nm.

For all animal groups (mAb, $F(ab')_2$ and Fab) examined with either FMT/CT and PET/MRI, linear functions of blood antibody levels were calculated based on the concentration levels determined by ECLIA. The time point x of the IC50 level (y=50) was determined.

Analysis of Anti-EGFR Expression and Anti-EGFR Antibody Binding

In order to confirm the in vivo EGFR expression of A-431 tumor cells and anti-EGFR antibody binding to EGFR receptors, fluorescence microscopy of tumors explanted from mice that were injected i.v. with labeled anti-EGFR constructs was performed. Tumor tissue sections of 1.5 µm thickness were scanned with a slide scanner (Pannoramic 250 Flash, 3D Histech, Budapest, Hungary) and binding of Alexa750-labeled anti-EGFR-constructs was investigated on a cellular level. In Supplemental

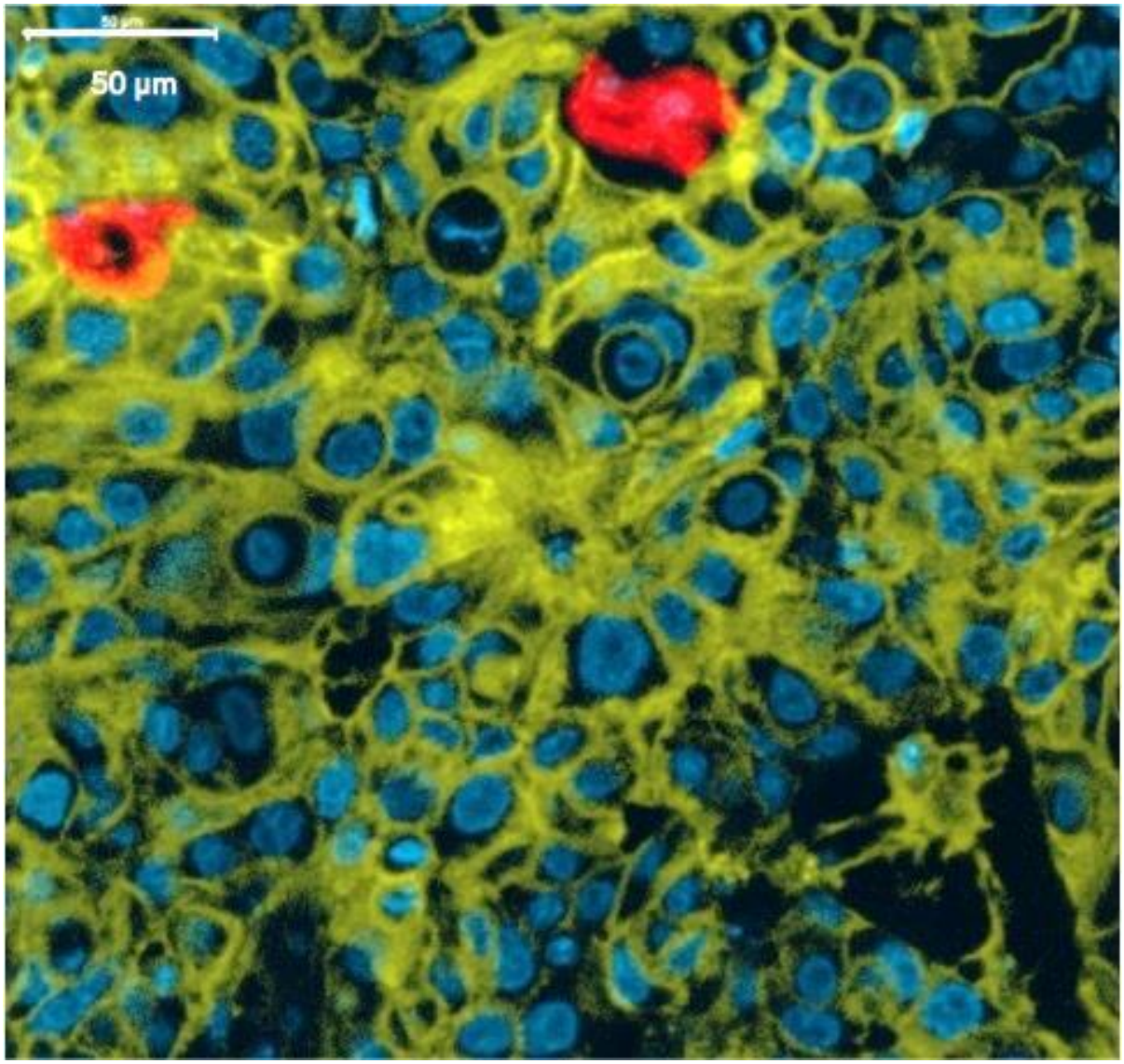
Figure 1, a representative image is shown indicating a strong binding of anti-EGFR-mAb-Alexa750 to the cell surface of A-431 tumor cells.

Phantom Experiments with FMT/CT

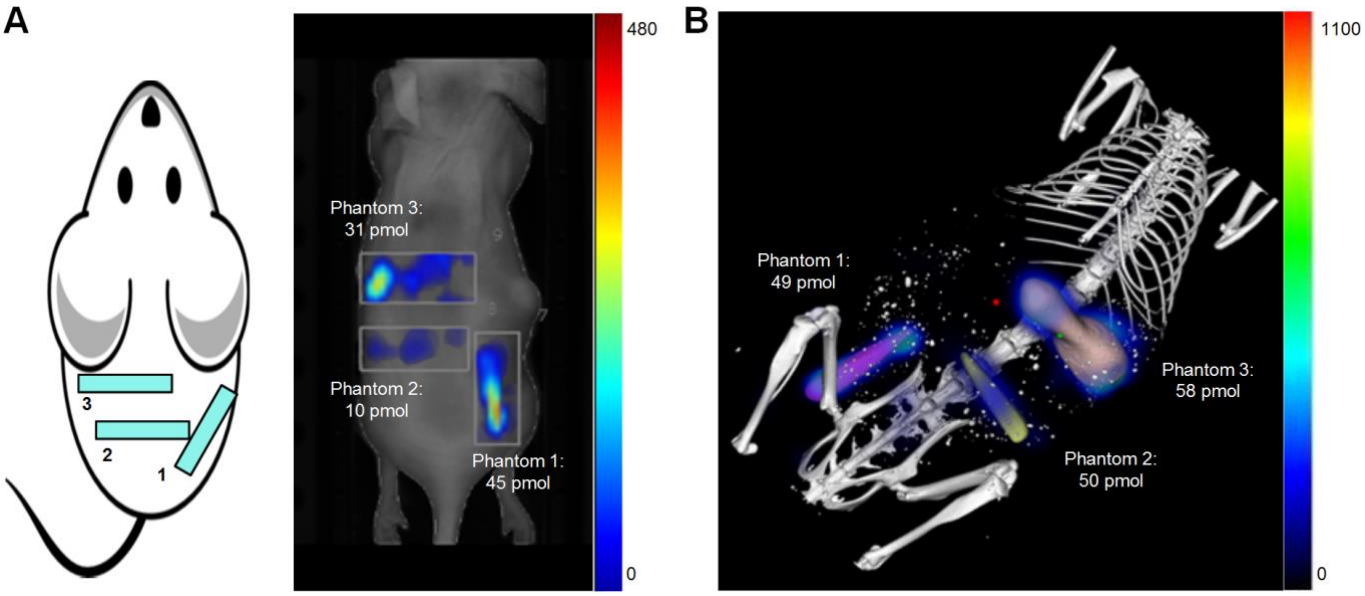
Prior to the *in vivo* biodistribution study phantom experiments were performed in order to verify the reliability of the fluorescence quantification via FMT (Supplemental Figure 2). Phantoms, filled with 100 µl of Alexa750-labeled antibody (50 pmol), were placed subcutaneously (phantom 1) and into the mouse abdomen (phantoms 2, 3). The fluorescence signals of the phantoms were measured by FMT and quantified by FMT standard software (TrueQuant). In addition, the results of the FMT standard reconstruction were compared to the improved reconstruction using FMT/CT datasets and the algorithm considering absorption and scattering effects of the tissues [11]. The quantification via FMT standard reconstruction of fluorescent phantoms leads to an underestimation of the true values (see Supplemental Figure 2A). Furthermore, the reconstructed quantitative values of the phantoms placed in the abdomen (phantom 2 and 3) were strongly influenced by the position of the phantoms. The improved reconstruction of fused FMT/CT datasets, however, generated more reliable fluorescence quantitation (Phantom 1: 49 pmol, Phantom 2: 50 pmol, Phantom 3: 58 pmol) (see Supplemental Figure 2B).

In Vivo Biodistribution after 24 h

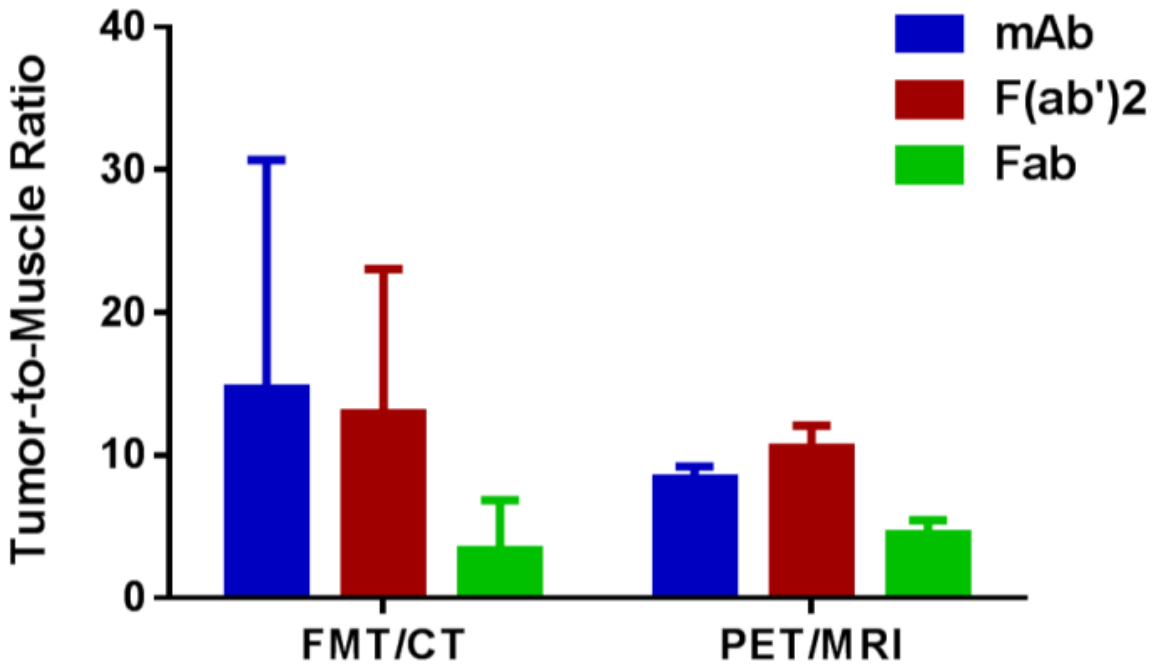
In order to report the signal-to-noise ratio of the *in vivo* measurements, the tumor uptake-to-muscle ratio was calculated. This is shown for both *in vivo* imaging technologies.



Supplemental Figure 1: Binding of anti-EGFR-mAb-Alexa750 to A-431 tumor cells 24 hours after injection. Representative section of the tumor showing strong binding of anti-EGFR-mAb-Alexa750 to A-431 tumor on a cellular level (blue = cell nuclei, yellow = anti-EFGR-mAb-Alexa750, red = vessel)."



Supplemental Figure 2: Quantification of fluorescence phantoms placed in a mouse post-mortem. A Positions of three phantoms placed in a mouse and results of FMT measurement analyzed by FMT standard reconstruction. **B** Results of FMT/CT measurement analyzed by an improved reconstruction algorithm. The image shows a 3D overlay of the mouse bone structure with segmented phantom areas and fluorescence signals. The segmented phantoms are illustrated in pink, yellow and orange.



Supplemental Figure 3: Tumor-to-muscle ratio of %ID/cm³ values measured after 24 hours by FMT/CT or PET/MRI. The ratio of the quantitative values of tumor and muscle uptake of each mouse 24 hours after injection was determined and mean values for each group are shown in a bar chart.

Supplemental Table 1: Quantitative biodistribution values [Mean +/- SD in %ID/cm³] of anti-EGFR compounds (mAb, F(ab')$_2$, Fab) measured by FMT/CT and PET/MRI after two hours.

2 hours	FMT/CT			PET/MRI		
	mAb	F(ab')$_2$	Fab	mAb	F(ab')$_2$	Fab
Liver	34.4 +/- 9.4	40.1 +/- 6.5	32,1+/- 5.7	7.8 +/- 1.6	5.3 +/- 0.5	2.3 +/- 0.5
Kidneys	7.9 +/- 5.4	22.9 +/- 9.2	52.7 +/- 11.2	5,6 +/- 1	30.4 +/- 3.9	64.2 +/- 8.1
Muscle	2.8 +/- 2	2.3 +/- 2.9	1.5 +/- 1.8	0.9 +/- 0.2	0.6 +/- 0.2	0.4 +/- 0.4
Tumor	10.9 +/- 3.6	4.8 +/- 3	8.1 +/- 5.8	1.9 +/- 5.9	2.1 +/- 0.5	1.1 +/- 0.6

Supplemental Table 2: Quantitative biodistribution values [Mean +/- SD in %ID/cm³] of anti-EGFR compounds (mAb, F(ab')$_2$, Fab) measured by FMT/CT and PET/MRI after 24 hours.

24 hours	FMT/CT			PET/MRI		
	mAb	F(ab')$_2$	Fab	mAb	F(ab')$_2$	Fab
Liver	20.9 +/- 2.5	21.3 +/- 4.5	7.5 +/- 3	5.4 +/- 0.5	3.6 +/- 0.5	3.5 +/- 0.4
Kidneys	3.9 +/- 1.3	7.8 +/- 4	7.1 +/- 3.6	3.5 +/- 0.3	21.1 +/- 1.4	24.3 +/- 1.6
Muscle	1.9 +/- 2.1	0.9 +/- 1.5	0.3 +/- 0.3	0.9 +/- 0.1	0.4 +/- 0.1	0.3 +/- 0.03
Tumor	13.1 +/- 2.5	3.7 +/- 1.7	1.9 +/- 1.9	7.3 +/- 1.2	3.7 +/- 0.7	1.2 +/- 0.16