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Improved micro-distribution of antibody-photon absorber conjugates after initial near infrared photoimmunotherapy (NIR-PIT)

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Abstract

Near infrared photoimmunotherapy (NIR-PIT), a targeted cancer therapy which uses an antibody-photon absorber conjugate (APC) and near infrared light exposure, dramatically improves nano-drug delivery into treated tumor beds due to enhanced vascular permeability. We investigated the micro-distribution of APCs in a variety of NIR-PIT treated tumors. Either Cetuximab (cet) or Trastuzumab (tra) conjugated with IR700 (cet-tra-IR700) was administered, as appropriate, to each mouse model of tumor. Tumor-bearing mice implanted with A431-GFP, MDAMB468-GFP, 3T3Her2-GFP or N87-GFP were separated into 5 groups: group 1 = no treatment; group 2 = cet-tra-IR700 i.v.; no light exposure group 3 = cet-tra-IR700 i.v., NIR light exposure; group 4 = cet-tra-IR700 i.v. and additional cet-tra-IR700 i.v. at 24 hours but no light exposure; group 5 = cet-tra-IR700 i.v., NIR light exposure and additional cet-tra-IR700 i.v. immediately after NIR but no additional NIR light exposure. *In vivo*, *ex vivo* and microscopic fluorescence imaging was performed. Fluorescence from the surface of the tumor (s-tumor) was compared to fluorescence from deeper areas of the tumor (d-tumor). In general, there was no significant difference in the fluorescence intensity of GFP in the tumors among all groups, however the highest IR700 fluorescence intensity was consistently shown in group 5 tumors due to added APC after NIR-PIT. Fluorescence microscopy in all tumor types demonstrated that GFP relative fluorescence intensity (RFI) in s-tumor was significantly lower in group 3 and 5 (NIR-PIT groups) than in group 1, 2, and 4 (no NIR-PIT) yet there was no significant difference in d-tumor RFI among all groups. IR700 fluorescent RFI in the d-tumor was highest in group 5 (NIR-PIT +additional APC) compared to the other groups. Cell killing after NIR-PIT was primarily on the surface, however, APCs administered immediately after NIR-PIT penetrated deeper into tissue resulting in improved cell killing after a 2nd NIR-PIT session. This phenomenon is explained by increased vascular permeability immediately after NIR-PIT.

Graphical abstract

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Keywords

photoimmunotherapy; enhanced permeability and retention effect; drug delivery; micro distribution; near infrared light

1. Introduction

The ability to deliver drugs to cancers has a marked effect on the effectiveness of therapy. Anticancer drugs often fail because heterogeneous vascularity, increased interstitial pressure and structural barriers imposed by the extracellular matrix prevent the drug from reaching its target in sufficient concentrations to be effective [1,2]. Therefore, methods to enhance drug delivery are being actively pursued.

Delivery of nano-sized drugs is particularly problematic. Nano-sized agents, such as monoclonal antibodies, tend to circulate for longer times and are often removed from the circulation by the liver, preventing the drug from ever reaching its target. The delivery of nano-sized agents to tumors largely relies on the intrinsically leaky nature of tumor vessels compared with healthy vessels in normal organs. This is known as the enhanced permeability and retention (EPR) effect [3]. The EPR effect results in modest improvements in drug delivery into tumors compared to normal tissue, but the effect is relatively small [4]. Therefore, new methods which can enhance delivery beyond the EPR effect are being explored.

Near infrared photoimmunotherapy (NIR-PIT) is a newly developed cancer treatment that employs a targeted monoclonal antibody-photon absorber conjugate (APC), IRDye700DX (IR700, silica-phthalocyanine dye) [5]. Within minutes of exposure to NIR, cells previously exposed to an APC, rapidly increase in volume leading to rupture of the cell membrane, and extrusion of cell contents into the extracellular space. Because the diffusion of the APC beyond the vessel is limited, it is maximally bound to cells in the immediate perivascular space. Rapid cell killing of perivascular tumor leads to an immediate increase in vascular permeability, allowing the leakage of nano-sized particles into the tumor space up to a 24-fold compared to non-treated tumors. This dramatic increase in permeability, and their subsequent retention in NIR-PIT treated tumors, has been termed super enhanced permeability and retention (SUPR) [6–8]. SUPR effects have also been reported to enable

the homogeneous redistribution of circulating or reinjected APCs and other nano-sized agents within treated tumor beds after NIR-PIT, presenting the possibility of improving therapeutic effects with additional exposures of NIR-light as more APC leaks into the tumor [7]. The deeper distribution of APCs after initial NIR-PIT is ideal for effective second NIR light exposures. However, the micro-distribution of APCs in the tumor after NIR-PIT has not been studied across multiple cell types and APC types. Herein, we investigate the micro-distribution of APCs after NIR-PIT using four different animal tumor models with two different APCs, cetuximab-IR700 and trastuzumab-IR700.

2. Materials and methods

2.1. Reagents

Water soluble, silica-phthalocyanine derivative, IRDye 700DX NHS ester was obtained from LI-COR Biosciences (Lincoln, NE, USA). Cetuximab, a chimeric (mouse/human) IgG₁ mAb directed against EGFR, was purchased from Bristol-Meyers Squibb Co (Princeton, NJ, USA). Trastuzumab, 95% humanized IgG₁ mAb directed against HER2, was purchased from Genentech (South San Francisco, CA, USA). All other chemicals were of reagent grade.

2.2. Synthesis of IR700-conjugated cetuximab and trastuzumab

Conjugation of dyes with monoclonal antibody was performed according to previous reports [5]. In brief, cetuximab or trastuzumab (1.0 mg, 6.8 nmol) was incubated with IR700 NHS ester (60.2 µg, 30.8 nmol) in 0.1 M Na₂HPO₄ (pH 8.6) at room temperature for 1 h. The mixture was purified with a Sephadex G25 column (PD-10; GE Healthcare, Piscataway, NJ, USA). The protein concentration was determined with the Coomassie Plus protein assay kit (Thermo Fisher Scientific Inc, Rockford, IL, USA) by measuring the absorption at 595 nm with UV-Vis (8453 Value System; Agilent Technologies, Santa Clara, CA, USA). The concentration of IR700 was measured by absorption at 689 nm to confirm the number of fluorophore molecules per mAb. The synthesis was controlled so that an average of two IR700 molecules was bound to a single antibody. We abbreviate IR700 conjugated to cetuximab as cet-IR700, and to trastuzumab as tra-IR700.

2.3. Cell culture

EGFR-expressing A431-GFP, MDAMB468-GFP and HER2-expressing 3T3/Her2-GFP cells stably expressing GFP were established by our laboratory. HER2-expressing N87-GFP cell stably expressing GFP were purchased from ANTI CANCER (San Diego, CA, USA). High expression GFP was confirmed in the absence of a selection agent with 10 passages. Cells were grown in RPMI 1640 (Life Technologies, Gaithersburg, MD, USA) supplemented with 10% fetal bovine serum and 1% penicillin/streptomycin (Life Technologies) in tissue culture flasks in a humidified incubator at 37 °C at an atmosphere of 95% air and 5% carbon dioxide.

2.4. Animal and tumor models

All *in vivo* procedures were conducted in compliance with the Guide for the Care and Use of Laboratory Animal Resources (1996), US National Research Council, and approved by the

local Animal Care and Use Committee. Six- to eight-week-old female homozygote athymic nude mice were purchased from Charles River (NCI-Frederick, Frederick, MD). Each GFP transfected cell line (A431-GFP; 2×10^6 , MDAMB468; 6×10^6 , 3T3Her2-GFP; 2×10^6 , N87-GFP; 1×10^7) was injected subcutaneously in the dorsa of the mice under isoflurane anesthesia. Tumors were studied after they reached volumes of approximately 100 mm³.

2.5. Treatment regimens

We used cet-IR700 as the agent for A431-GFP and MDAMB468 tumor bearing mice, and tra-IR700 for 3T3Her2-GFP and N87-GFP tumor bearing mice. Tumor bearing mice were randomized into 5 groups of at least 7 animals per group for the following treatments: group 1 = no treatment (control); group 2 = 100 µg of cet or tra-IR700 i.v., no NIR light exposure (i.v.); group 3 = 100 µg of cet or tra-IR700 i.v., NIR light was administered at 50 J/cm² on day 1 after injection (PIT); group 4 = 100 µg of cet or tra-IR700 i.v. and additional 50 µg of cet or tra-IR700 i.v. on day 1, no NIR light exposure (i.v. + additional i.v.); group 5 = 100 µg of cet or tra-IR700 i.v., NIR light was administered at 50 J/cm² on day 1 after injection and 50 µg of cet or tra-IR700 i.v. immediately after NIR-PIT (SUPR).

2.6. Imaging

In vivo fluorescence imaging was performed for A431-GFP tumor bearing mice [7]. All mice were euthanized with carbon dioxide on day 2 and tumors were extracted (Figure 1A). *Ex vivo* fluorescence imaging was performed for A431-GFP tumors. Additionally, microscopic fluorescence imaging was performed for all cell lines. We abbreviate fluorescence signal derived from GFP as GFP-signal and that from IR700 as IR700-signal.

2.6.1. In vivo fluorescence imaging using A431-GFP tumor bearing mice—

Serial fluorescence images were assessed before and after NIR light exposure (day 1 and day 2) (Figure 1A) using a Pearl Imager (LI-COR Biosciences, Lincoln, Nebraska, USA) with a 700 nm fluorescence channel. A region of interest (ROI) was placed on the tumor and the average fluorescence intensity of IR700-signal was calculated for each ROI using Pearl Cam Software (LICOR Biosciences).

2.6.2. Ex vivo fluorescence imaging—*Ex vivo* fluorescence imaging was obtained with the Pearl Imager for detection of IR700-signal and the Maestro Imager (Cri, Woburn, MA, USA) was obtained for GFP-signal. For GFP imaging a band-pass excitation filter from 445 to 490 nm and a long-pass emission filter over 515 nm were used. The tunable emission filter was automatically increased in 10-nm increments from 515 to 580 nm at a constant exposure time (250 msec). The spectral fluorescence images consisted of autofluorescence spectra and spectra from GFP, which were then unmixed from each other based on the characteristic spectral pattern of GFP using Maestro software (CRi). ROIs were placed on the tumor in the GFP images and the average fluorescence intensity value was measured for each ROI.

2.6.3. Fluorescence microscopy—Extracted tumors were frozen with OCT compound (SAKURA Finetek Japan Co., Tokyo, Japan) and frozen sections (10 µm thick) were prepared followed by fluorescence microscopy using the BX61 (Olympus America, Inc.,

Melville, NY, USA) equipped with the following filters; excitation wavelength 460 to 490 nm, 590 to 650 nm, emission wavelength 510 to 550 nm, 665 to 740 nm long pass for GFP- and IR700-signal, respectively. Transmitted light differential interference contrast (DIC) images were also acquired. First, we defined the area within 500 μm from tumor surface as s-tumor and the area over 500 μm from tumor surface as d-tumor on fluorescence microscopic images. We evaluated fluorescence signal from s-tumor and from d-tumor portions of the tumor using different histologic sections. ROIs were placed on s-tumor, d-tumor and background, and average fluorescence intensity was calculated for each ROI using the Image J software (<http://rsb.info.nih.gov/ij/>). Next, relative fluorescence intensity (RFI) was calculated using the equation: $\text{RFI} = \text{average fluorescence intensity of tumor (s- or d-tumor)} / \text{average fluorescence intensity of background}$.

2.7. Second NIR light exposure

A431-GFP tumor bearing mice were randomized into 2 groups of at least 10 animals per group for the following treatments: group 5a = 100 μg of cet-IR700 i.v., NIR light was administered at 50 J/cm^2 on day 1 after injection and 50 μg of cet-IR700 i.v. immediately after NIR-PIT (SUPR); group 5b = 100 μg of cet-IR700 i.v., NIR light was administered at 50 J/cm^2 on day 1 after injection and 50 μg of cet-IR700 i.v. immediately after NIR-PIT and additional 50 J/cm^2 of light on day 3 (SUPR + NIR). All mice were euthanized with carbon dioxide on day 3 and tumors were extracted (Supplementary Figure 1A). Extracted tumors were frozen and frozen sections (10 μm thick) were prepared followed by fluorescence microscopy using the BX61.

2.8. Statistical analysis

Statistical analyses were carried out using GraphPad (GraphPad Prism; GraphPad Software, La Jolla, CA, USA). For multiple comparisons, a one-way analysis of variance (ANOVA) followed by the Bonferroni's multiple comparisons test was used. P -value of <0.05 was considered statistically significant.

3. Results

3.1. *In vivo* fluorescence imaging using A431-GFP tumor

On day 1 the tumor in groups 2–5 showed higher IR700 fluorescence intensity than the tumor in group 1. In the tumors of groups 3 and 5 (NIR-PIT), IR700 fluorescence intensity decreased after NIR light exposure. On the other hand IR700 fluorescence was preserved in groups 2 and 4 (no NIR-PIT), and fluorescence of the tumor in group 4 was higher than that in group 2 due to the higher dose of APC at 24 hours. However, IR700 fluorescence was even higher in group 5 tumors (NIR-PIT and immediate additional APC) compared to group 2 and 4 tumors on day 2 due to NIR-PIT-induced vascular leakage (Figure 1B).

3.2. *Ex vivo* fluorescence studies of A431-GFP tumor

There was no significant difference in GFP-signal among all groups (Figure 2A and 2B). The IR700-signal of group 3 (NIR-PIT) tumors was lower than group 2 or group 4 tumors (no NIR-PIT) ($p < 0.01$ and < 0.0001 , respectively) (Figure 2C). The highest IR700

fluorescence intensity among all groups was achieved in group 5 tumors (NIR-PIT with additional APC) (all p values < 0.0001) (Figure 2C).

3.3. Fluorescence microscopy of A431-GFP tumors

GFP-signal in group 1, 2 and 4 tumors showed homogenous GFP fluorescence whereas the GFP fluorescence pattern in group 3 and 5 tumors was heterogeneous due to the effects of NIR-PIT. Moreover, s-tumor fluorescence was lower than d-tumor fluorescence in NIR-PIT treated tumors (Figure 3).

The RFI of s-tumor was also significantly lower in group 3 and 5 tumors than in group 1, 2, and 4 tumors ($p < 0.01$) (Figure 4B), although there was no significant difference in the fluorescence of d-tumor among all groups (Figure 4C).

The IR700-signal fluorescence intensity of d-tumor was higher in group 5 tumors compared to tumors in other groups (Figure 3). IR700 RFI in s-tumor was significantly higher in group 4 tumors than in other groups ($p < 0.01$) (Figure 5B). IR700 RFI in d-tumor was significantly higher in group 5 tumors compared to tumors in other groups (all p values < 0.0001) (Figure 5C).

3.4. Second NIR light exposure

GFP-signal in group 5a tumors showed heterogeneous GFP fluorescence whereas the GFP fluorescence in group 5b tumors decreased in the whole tumor due to the effects of additional NIR-PIT. Tumor of group 5a showed relatively high IR700 signal in d-tumor compared with group 5b (Supplementary Figure 1B). There was no significant difference in GFP RFI in s-tumor among groups (Supplementary Figure 1C), yet the GFP RFI of d-tumor was significantly lower in group 5b tumors than in group 5a tumors ($p < 0.01$) (Supplementary Figure 1D).

3.5. Fluorescence microscopy of MDAMB468, 3T3Her2 and N87 tumors

3.5.1. MDAMB468-GFP tumor—GFP-signal and GFP RFI of s-tumor showed lower fluorescence signal in group 3 and 5 tumors due to treatment effects compared to untreated groups (Groups 1, 2, and 4) ($p < 0.01$) (Figure 6A and Supplementary Figure 2A), although there was no significant difference in d-tumor among all groups (Supplementary Figure 2B).

IR700-signal RFI in group 4 tumors was highest among all groups in s-tumor as there was no photobleaching effects in this group ($p < 0.05$) (Figure 6B), and IR700 RFI in group 5 tumors was highest among all groups in d-tumor due to the deeper penetration of the additional APC ($p < 0.05$) (Figure 6C).

3.5.2. 3T3Her2-GFP tumor—3T3Her2-GFP tumor demonstrated homogeneous GFP-signal in group 1, 2, and 4 tumors, and heterogeneous, (s-tumor-low, d-tumor-high), in group 3 and 5 tumors due to treatment effects (Figure 7A). The GFP RFI of s-tumor was significantly lower in group 3 and 5 tumors than in group 1, 2, and 4 tumors due to treatment effects ($p < 0.01$) (Supplementary Figure 2C). There was no significant difference in GFP RFI in d-tumor among groups (Supplementary Figure 2D). The IR700 RFI of s-tumors in

group 2 and 4 was higher than in group 1, 3, and 5 tumors because APC was administered but there was no photobleaching due to NIR-PIT ($p < 0.05$ and $p < 0.0001$, respectively) (Figure 7B), and the IR700-RFI of d-tumor was highest in group 5 tumors ($p < 0.0001$) (Figure 7C).

3.5.3. N87-GFP tumors—N87- GFP fluorescence of s-tumor was lower in group 3 and 5 tumors than in group 1, 2 and 4 tumors (Figure 8A). The RFI of GFP in s-tumor was lower in group 3 and 5 tumors than in group 1, 2, and 4 tumors ($p < 0.01$) (Supplementary Figure 2E). There was no difference in d-tumor RFI among all groups (Supplementary Figure 2F). The IR700 RFI of s-tumor in group 4 tumors was higher compared to all other groups ($p < 0.0001$) (Figure 8B). The d-tumor RFI was higher in group 4 tumors compared to group 1 and 3 tumors ($p < 0.05$), but the highest IR700 RFI was seen in group 5 tumors (all p values < 0.0001) (Figure 8C).

4. Discussion

A major design goal of intravenous therapies is that they be delivered in sufficient concentration to all parts of the tumor. This study shows that after NIR-PIT an additional dose of APC was able to distribute more fully throughout the treated tumor than was possible before NIR-PIT. This finding was observed across four different types of tumors and two different APC types indicating that it is a general property of NIR-PIT. This supports the concept that NIR-PIT, regardless of tumor type or antibody, leads to increased vascular permeability allowing better micro-distribution of subsequent injections of additional APC.

This study differs from prior studies on the treatment effects of NIR-PIT in several respects. It tests four different cell lines and two different antibodies. It also employs GFP as a surrogate of treatment and IR700 as a surrogate of APC accumulation. Finally, it evaluates these signals as a function of depth within the tumor. GFP signals were lower in the surface of tumors treated with NIR-PIT due to cell killing. However, the GFP signals were uniform in both treatment (groups 3 and 5) and non-treatment (groups 1, 2 and 4) in deeper parts of the tumor. This is likely due to the limited penetration of the initial APC injection and thus limited damage to deeper parts of the tumor. Therefore the deeper parts of the tumor were not treated in this study. Previous work has shown that repeated treatments of NIR-PIT result in deeper, more complete cell killing due to improved intra-tumoral distribution of existing or additional APCs due to enhancement of permeability and retention [7] that was also demonstrate in a second light exposure to group 5b tumors in this study (Supplementary Figure 1).

The GFP signal of the surface of the tumor was significantly lower in NIR-PIT-treated groups than in groups that did not undergo NIR-PIT likely owing to surface cell killing. Although NIR light physically is able to penetrate several centimeters into tissue [9], the therapeutic effects induced by NIR-PIT depends on the amount of APC deposited in the tumor, therefore surface tumor was preferentially killed. Thus, the lower concentration of APC deeper in the tissue accounts for the loss of GFP signal on the surface of the tumor.

The IR700 signal reflects the concentration of APC within the tumor. The highest IR700 fluorescence intensity on *ex vivo* and *in vivo* imaging was shown in tumors in group 5 that received a 2nd dose of APC following NIR-PIT. These results suggest that an additional immediate injection of APC after NIR-PIT leads to deeper tissue penetration of the APC and more complete treatment on subsequent NIR-PIT treatments.

It is well known that improving drug delivery is a key to therapeutic success. One method of improving drug delivery is to improve vascularity. For instance, radiation therapy primarily damages cancer cells with a much less pronounced effect on the vasculature. Nano-sized molecules can enter radiation treated tumors at a rate 2.2-fold higher than unirradiated tissue [10]. Light therapy with conventional photodynamic therapy (PDT) can also enhance the EPR effect up to 3-fold compared with control tumor, although this effect is limited to within 0 and 12 hours after PDT [11–13]. Because PDT causes damage to both tumor vasculature and the tumor vascularity, there is a danger that PDT can reduce vascularity, thus negatively affecting drug delivery [14, 15]. In contrast, NIR-PIT specifically kills APC-binding cancer cells by inducing immediate necrotic cell death without damaging normal cells in the immediate vicinity, including the vascular endothelium, leading to increases in vascularity and flow. This super enhanced permeability and retention that has been shortened to SUPR induced by NIR-PIT can increase delivery of nano-sized drugs by 24 fold compared to that by radiation therapy (2.2 fold) or PDT (3 fold). In this study, SUPR enabled deeper penetration of APC within the tumor.

Usually, naturally occurring tumors are phenotypically and functionally heterogeneous [16, 17]. Repeated NIR-PIT followed by reinjections of APC have been shown to be an effective strategy for cancer treatment in a mouse model [18]. However, xenograft models are generally more homogeneous with respect to antigen expression than spontaneously occurring tumors. In that case, instead of following NIR-PIT with additional APC, a good strategy might be to deliver higher doses of non-targeted anticancer drugs to the viable part of the NIR-PIT treated tumor as a method of improving effectiveness [8]. The effects of SUPR would tend to turn these non-targeted agents into targeted agents based on differential vascular leakiness.

In this study, we carefully performed serial analysis of histopathology for simultaneously detecting changes of tumor cell death and APC delivery after NIR-PIT in subcutaneously implanted tumors which expressed GFP as a marker. In order to observe these changes with superior spatial and temporal resolution, a recently established powerful technology of *in vivo* fluorescence imaging [19, 20] using cell-cycle responsible fluorescent protein labelled tumor cells [21–23] might be able to simultaneously monitor cell death and APC delivery in real time in living mice. However, a limited field of view of the *in vivo* fluorescence imaging technology might hamper for simultaneously observing superficial and deep parts of a tumor. Another caveat in this study is the use of subcutaneously xenografted human tumors in athymic mice that does not fully represent human cancers. Superior tumor models including surgically implanted patient-derived orthotopic tumor models can simulate cancers in patients better than xenografted tumor models [24, 25], yet consistently growth of surgically implanted orthotopic tumors requires highly trained surgical skills. Therefore, we will use such orthotopic tumor models in our future studies.

Conclusions

The distribution of antibody-photon absorber conjugates was improved after NIR-PIT as demonstrated by deeper penetration of the conjugate within the tumor. This finding may explain the enhanced effectiveness of multiple sequential administrations of conjugates and light therapy.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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Abbreviations

APC	antibody- photon absorber conjugate
cet	cetuximab
EPR	enhanced permeability and retention
d-tumor	deep area of tumor
GFP	green fluorescent protein
HER2	human epidermal growth factor receptor type 2
IR700	IRDye700DX
i.v.	intra venous
LED	light-emitting diode
NIR	near-infrared
PDT	photodynamic therapy
PIT	photoimmunotherapy
RFI	relative fluorescence intensity
ROI	regions of interest
s-tumor	surface area of the tumor
SUPR	super enhanced permeability and retention
tra	trastuzumab

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Fig. 1. *In vivo* fluorescence imaging using A431-GFP tumor

(A) Treatment regimen. Fluorescence images were obtained at each time point as indicated.

(B) *In vivo* and *ex vivo* fluorescence imaging of A431-GFP tumor. After exposure of NIR light, IR700 fluorescence signal decreased in group 3 and 5, but the IR700 fluorescence signal was preserved in group 4 even at day 2. On day 2 the tumor of group 5 showed the highest IR700 fluorescence signal.

Fig. 2. Ex vivo fluorescence studies of A431-GFP tumor

(A) White light images (left), GFP fluorescence images (middle) and IR700 fluorescence images (right) of A431-GFP tumors. (B) Fluorescence intensity value of GFP on ex vivo A431-GFP tumors. There were no significant differences among the groups. (C) Fluorescence intensity value of IR700 on ex vivo A431-GFP tumors. Tumors of group 2 and 4 showed significantly higher fluorescence intensity value than that of the control group ($*p < 0.01$ and <0.0001 , respectively). However, tumors of group 5 showed the highest fluorescence intensity among all groups ($****p < 0.0001$).

Fig. 3. Fluorescence microscopy of A431-GFP tumor

DIC (left), GFP fluorescence- (middle) and IR700 fluorescence microscopic images (right) of A431-GFP tumors. GFP fluorescence signal in tumors of group 2 and 4 was relatively homogeneous, but in group 3 and 5 tumors was more heterogeneous, with low signal in the surface of the tumor, s-tumor. IR700 fluorescence signal of d-tumor showed relatively high signal in group 5 compared with other groups. Scale bars = 100 μ m.

Fig. 4. Quantification of histological GFP fluorescence intensity

(A) Regions of interest (ROI) were placed on the background (a), surface area of tumor (b) and deep area of tumor (c). (B) RFI of GFP-signal of s-tumor. RFI was significantly lower in group 3 and 5 than that in group 1, 2 and 4 (** $p < 0.01$). (C) RFI of GFP-signal of d-tumor. There were no significant differences among all groups.

Fig. 5. Quantification of histological IR700 fluorescence intensity

(A) Regions of interest (ROI) were placed on the background (a), surface area of tumor (b) and deep area of tumor (c). (B) RFI of IR700-signal of s-tumor. RFI was significantly higher in group 4 than that in other groups (** $p < 0.01$). (C) RFI of IR700-signal of d-tumor. RFI of group 5 was highest among all groups (** $p < 0.0001$).

Fig. 6. Fluorescence microscopy of MDAMB468-GFP tumors

(A) DIC (left), GFP fluorescence- (middle) and IR700 fluorescence microscopic images (right) of MDAMB468-GFP tumors. In tumors of group 3 and 5 fluorescence signal of s-tumor was relatively low compared to that of d-tumor. Scale bars = 100 µm. (B) RFI of IR700-signal of s-tumor. RFI in group 4 was higher than in group 1 and 3 (* $p < 0.05$). (C) RFI of IR700-signal of d-tumor. RFI in group 5 was highest among all groups (** $p < 0.05$).

Fig. 7. Fluorescence microscopy of 3T3Her2-GFP tumors

(A) DIC (left), GFP fluorescence- (middle) and IR700 fluorescence microscopic images (right) of 3T3Her2-GFP tumors. GFP fluorescence signal in tumor of group 1, 2 and 4 was relatively homogeneous, but group 3 and 5 tumors were more heterogeneous as noted in their lower s-tumor signal. Tumors of group 5 showed relatively high IR700 signal in d-tumor compared with other groups. Scale bars = 100 µm. (B) RFI of IR700-signal of s-tumor. RFI of group 2 and 4 was higher than that of group 1, 3, and 5 ($*p < 0.05$ and $p < 0.0001$, respectively). (C) RFI of IR700-signal of d-tumor. RFI of group 5 was highest among all groups ($***p < 0.0001$).

Fig. 8. Fluorescence microscopy of N87-GFP tumors

(A) DIC (left), GFP fluorescence- (middle) and IR700 fluorescence microscopic images (right) of N87-GFP tumors. GFP fluorescence signal of s-tumor in group 3 and 5 was lower than in group 1, 2 and 4. Tumor of group 5 showed relatively high IR700 signal in d-tumor compared with other groups. Scale bars = 100 µm. (B) RFI of IR700-signal of s-tumor. RFI in group 4 was highest among all groups (** $p < 0.0001$). (C) RFI of IR700-signal of d-tumor. RFI was significantly higher in group 4 than that of group 1, 2, and 3 (** $p < 0.05$), but was highest in group 5 among all groups (**** $p < 0.0001$).