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Tumor-specific labeling of pancreatic cancer using a humanized anti-CEA antibody conjugated to a near-infrared fluorophore

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Abstract

Background/Purpose—Development of a humanized fluorophore-conjugated antibody that can improve contrast for fluorescence guided oncologic surgeries.

Methods—BxPC-3-GFP pancreatic cancer cells were injected into flanks of nude mice. Fragments of subcutaneous tumors were grafted onto the pancreatic tail of recipient mice to create orthotopic xenograft models of pancreatic cancer. After tumors developed for 4 weeks, a humanized anti-CEA antibody conjugated to an 800 nm NIR fluorescent dye (hM5A-IR800), was injected intravenously. Mice were imaged at 6, 12, 24, 48, and 72 hours after injection.

Results—Fluorescence imaging showed that hM5A-IR800 specifically localized to BxPC-3 human pancreatic cancer cells. The fluorescent probe localized to cell surfaces *in-vitro* and specifically co-localized with GFP labeled tumors in an orthotopic pancreatic xenograft model *in-vivo*. Serial imaging at specific time points showed peak signal intensity of the orthotopic pancreatic tumor at 48 hours and this time point corresponded with a maximal tumor to background ratio of 21.23 at 48 hours.

Discussion/Conclusion—hM5A-IR800 was successfully able to specifically label orthotopic pancreatic tumors *in-situ*. The longer wavelength allowed deeper tissue penetration, particularly in tumor areas covered by normal pancreatic parenchyma. The probe had expected kinetics for an antibody-fluorophore conjugate with the peak signal intensity reached at 48 hours. There was a clear tumor signal with a tumor-to-background ratio (TBR) greater than 5 at all time points, with high contrast (TBR of 16.6) at 48 hours. hM5A-IR800 demonstrated excellent tumor localization

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and a very bright signal. It is a promising agent for future clinical fluorescence-guided surgery applications.

Introduction

Pancreatic cancer is an aggressive malignancy with a poor survival rate. The only curative treatment for pancreatic cancer is surgical resection with true negative margins. However, delineation of tumor margins is challenging in practice. Surgeons commonly use visual and tactile feedback along with clinical judgment based on pre-operative cross sectional imaging to determine the boundaries of resection. However, this is insufficient; as many as 80% of pancreatic resections are considered R1 (defined as the presence of cancer cells directly at, or less than 1mm, from the margin) and this remains an independent determinant of post-operative outcome (1,2). Fluorescence image guidance is an approach that can be utilized within the operative field to enhance contrast and visualization of the neoplasm.

Current utilization of fluorescence navigation technology within the operating room has been limited to non-specific dyes such as indocyanine green and as such, oncologic applications have been limited. Conjugation of tumor-targeting antibodies to fluorophores confers specificity to the dye and can help surgeons accordingly visualize tumor, margins, and any satellite lesions *in-situ*. Fluorescence-guided surgery (FGS) has been shown to significantly reduce the volume of residual cancer and extend disease-free and overall survival in a number of pre-clinical orthotopic mouse models of colon, pancreatic, breast, lung, brain, skin, and soft tissue cancers (3–12).

The human carcino-embryonic antigen (CEA) or CECAM5 is an appealing target as it is a well-characterized tumor antigen. It is present in low levels during human embryonic development, absent in normal adult tissue, and is highly expressed in a number of solid GI malignancies. Our lab has pioneered the use of anti-CEA antibodies to target a fluorescence signal in orthotopic mouse models of pancreatic and colon cancers. We have transitioned from proof-of-concept studies using murine anti-CEA antibodies conjugated to fluorophores, to chimeric antibodies (13,14). However, even chimeric antibodies have been shown to induce the production of human-anti-mouse antibodies (15).

In the present study, we used a humanized anti-CEA hT84.66-M5A (hM5A) monoclonal antibody (mAb) antibody developed by Yazaki et al (16). We chose to conjugate the antibody to an 800 nm NIR fluorescent dye. Previous work in our laboratory has shown that the use of fluorophores in the near-infrared range decreases auto-fluorescence and background signal (14). Here, we show that hM5A-IR800 probes can successfully target human pancreatic cancer *in-situ* using orthotopic xenograft mouse models, with useful kinetics and a favorable tumor-to-background ratio.

Methods

Tissue Culture

The human pancreatic cancer cell lines BxPC-3 (ATCC® CRL-1687™), stably expressing green fluorescent protein (GFP) and MiaPACA-2 (ATCC® CRL-1420™) expressing RFP,

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were maintained in Roswell Park Memorial Institute 1640 (RPMI-1640) medium (Gibco-BRL, Grand Island, NY). The medium was supplemented with 10% fetal calf serum (Hyclone, Logan, UT), 1% L-Glutamine, and 1% penicillin/streptomycin (Gibco-BRL). The cells were incubated at 37°C in a 5% CO2 incubator.

Antibody Conjugation

The humanized M5A mAb was developed by grafting the CDR region of the murine anti-CEA antibody mT84.66 onto the humanized anti-p185HER2 antibody (Trastuzumab) framework and expressed as previously described (16). The purified hM5A antibody was conjugated with NHS-IRDye800CW (LI-COR Biosciences, Lincoln, NE) at a 10-fold molar excess of the esterified dye at room temperature for 1 hour. Concentrations of the hM5A-IR800 conjugate were determined by $Absorbance_{280}$. The final concentration of antibody-dye conjugate was 5.7 mg/mL with an average of 2 dye molecules per IgG based on mass spectrometry.

Immunofluorescence Imaging

BxPC-3-GFP and MiaPaca-2-RFP cells were grown overnight on cover slips coated with 0.1% gelatin (5×10^5 cells per well). The cells were incubated with 200 nM of hM5A-IR800 for 1 hour at 37°C. After washing, the cells were fixed with 4% formaldehyde (Fisher Scientific, Waltham, MA) for 15 minutes and stained with 300 nM DAPI ((Fisher Scientific, Waltham, MA) for 5 minutes. The cover slips were mounted on slides with ProLong Gold antifade reagent (Life Technologies, Grand Island, NY). Cells were imaged at 20x with a Nikon A1R Confocal laser microscope (Nikon Instruments, Melville, NY).

Animal Care

Immunocompromised nude nu/fox mice were maintained in a barrier facility on high-efficiency particulate air (HEPA)-filtered racks at AntiCancer Inc. Mice were maintained ad lib on an autoclaved laboratory rodent diet (Teckland LM-485; Western Research Products, Orange, CA, USA) and kept on a 12 hour light/12 hour dark cycle. All surgical procedures and intravital imaging were performed with the animals anesthetized by intramuscular injection of an anesthetic cocktail composed of ketamine 100mg/kg (MWI Animal Health, Boise, ID), xylazine 10mg/kg (VWR, Brisbane, CA), and acepromazine 3 mg/kg (Sigma, Saint Louis, MO). All animal studies were conducted in accordance with the principles and procedures outlined in the NIH Guide for the Care and Use of Animals under PHS Assurance Number A3873-1.

In-Vivo Studies

BxPC-3-GFP pancreatic cancer cells (1×10^6 cells per animal) were injected subcutaneously into the flank of nude mice. The tumors were allowed to grow for 4 weeks or until 7-10mm in size. The tumors were harvested and 2 mm^3 fragments were implanted into the pancreatic tail of recipient nude mice to create orthotopic models of pancreatic cancer. After the tumors developed for 2 weeks, 75 μg of M5A-IR800 was injected via tail-vein. 3 mice per time point were sacrificed and imaged at 6, 12, 24, 48, 72, and 96 hours after injection using the Maestro CRI imaging system (Perkin Elmer, Waltham, MA). The images were acquired at

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the GFP wavelength (Excitation 488 nm, emission 510 nm) and IRDye800 wavelength (Excitation 778 nm, emission 800 nm). Fluorescence intensity was quantified at the skin and tumor after spectral unmixing using the Maestro CRI software. Fluorescence intensity was adjusted for background signal at the skin by subtracting the intensity value at an area of adjacent skin to the intensity value at the tumor. Tumor-to-background ratio (TBR) was calculated by dividing the intensity value at an area of adjacent skin to the intensity value at the tumor.

Results

Staining with hM5A-IR800 showed that the fluorescent antibody bound the CEA-expressing BxPC-3-GFP human pancreatic cancer cell line (Figure 1a). Merged images of the GFP and hM5A-IR800 channel show the fluorescent antibody localized at cell surfaces and within cells. Staining with hM5A-IR800 showed little to no antibody binding to the CEA-negative MiaPaCa-2-RFP human pancreatic cancer cells (Figure 1b). Merged images of the RFP and hM5A-IR800 channel show poor antibody localization.

Non-invasive imaging of mice at 48 hours showed that a fluorescence signal from the BxPC-3 pancreatic tumor was visible through the skin, abdominal wall soft tissue, and musculature (Figure 2a, 2b). When comparing the GFP and LICOR800 channels, the near-infrared wavelength demonstrates a stronger tumor signal through the tissue layers. In the same mouse, after laparotomy, the tumors are again visible in the GFP and LICOR-IR800 channels (Figure 2c, 2d). The areas labeled by hM5A-IR800 clearly overlap with the GFP-tagged tumor. Magnified views show that the fluorescence signal from GFP can be dampened by the overlying pancreatic parenchyma and vasculature (Figure 2e, 2f). In contrast to IR800 signal where the fluorescence was clearly detectable through overlying tissue (Figure 2b: bottom panels).

To determine the pharmacokinetics of this antibody-dye conjugate, mice underwent intra-vital imaging at 6, 12, 24, 48, 72, and 96 hours after injection. The results are summarized in Figure 3a. Peak fluorescence signal was obtained at 48 hours after initial injection. The mean fluorescence intensity value at 48 hours was 1695 counts compared to 6 hrs (1186 counts), 12 hrs (1204 counts), 24 hrs (1428 counts), 72 hrs (1046 counts), and 96 hrs (1022 counts). There was a high fluorescence signal in the liver from the earliest time point at 6 hours, but this signal rapidly decreased after 12 hours. The signal at the liver was the following: 6 hrs (1050 counts), 12 hrs (954counts), 24 hrs (493 counts), 48 hrs (2269 counts), 72 hrs (111 counts), and 96 hrs (113 counts). There was also some mild background fluorescence signal at the skin and the highest signals were within the first 24-48 hours and the signal decreased steadily thereafter. The signal at the skin was as follows: 6 hrs (194 counts), 12 hrs (146 counts), 24 hrs (170 counts), 48 hrs (103 counts), 72 hrs (69 counts), and 96 hrs (85 counts).

The time of peak signal intensity correlated with the maximal TBR at 48 hours (TBR=16.6) as shown in Figure 3b. There was a TBR greater than 5 at all time points: 6 hrs (TBR=6.3), 12 hrs (TBR=8.3), 24 hrs (TBR=8.8), 72 hrs (TBR=15.7), and 96 hrs (TBR=15.4). The time of peak signal intensity correlated with the maximal TBR at 48 hours.

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Serial intra-vital images are displayed in Figure 4. All images demonstrate a fluorescence signal targeted to the tumor at the IR800 wavelength (blue asterisks). The images show additional background noise at the liver (green arrows) and bladder (yellow arrowheads) that steadily decreases over time.

Discussion

In these experiments, we demonstrate the feasibility, kinetics, and advantages of using a humanized anti-CEA antibody conjugated to a near-infrared IR800 fluorophore, for *in-situ* tumor specific labeling of orthotopic pancreatic xenografts.

The BxPC-3 tumor was selected as a high CEA expressing human pancreatic cancer cell line as previously shown by flow cytometry (17,18). Cellular microscopy confirmed binding of the fluorescent antibody to this cell line *in-vitro* and its sub-cellular localization at the surface membrane, which is consistent with its known status as a cell surface glycoprotein. hM5A-IR800 is a probe that binds specifically to CEA as it did not demonstrate any binding at the same concentration in a low CEA expressing MiaPaca-2 pancreatic cancer cell line. Approximately 71-98% of pancreatic cancers are CEA positive on immunohistochemistry (19–21). However, in studies of immunohistochemical staining of CEA, there is no agreed upon threshold for determining positivity. A specimen can be considered positive when there is greater than 10% of the cells expressing the marker. Further studies are still needed to standardize a minimal number or percent of positive cells or serum levels needed for clinically relevant fluorescent surgical navigation

The anti-CEA humanized M5A is a versatile antibody for use in targeting a number of CEA-positive malignancies. In addition to showing promise as a probe for in-situ tumor-specific fluorescence delivery, the parental anti-CEA hT84.66-M5A antibody is being evaluated as a radioactive diagnostic imaging modality and as a therapeutic treatment. It is currently undergoing clinical trials for PET imaging conjugated with Cu-64 and for radiotherapy conjugated with Y-90 (NCT02293954, NCT00645060). Compared to previous work with M5A chimeric antibodies where nearly 30% of patients developed human-anti-chimera antibodies, no human anti-human antibody immunological response has thus far been detected in current studies using humanized M5A (Unpublished data courtesy of Dr P. Yazaki). In the present study, hM5A conjugated to a NIR-fluorophore becomes a highly specific probe for fluorescence in-situ tumor labeling.

In-vivo studies highlight the improvements in visualization using a near-infrared wavelength. GFP is a fluorescent protein in the visible wavelength that is subject to hemoglobin quenching. The fluorescence signal is limited with increasing tissue depth. The use of near-infrared fluorophores improves tissue penetration with decreased background noise. LICOR-IRDye800CW is a promising dye for future clinical use as it is a bio-compatible fluorophore in the near-infrared spectrum and it has been validated in several Phase I/II clinical trials for fluorescence-guided surgery (FGS) of head and neck cancer (NCT02415881, NCT01987375, NCT02736578) (22,23). Additionally, the emission and absorption spectra of LICOR-IRDye800CW overlap with that of indocyanine green (ICG).

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A number of FDA-approved imaging platforms exist for imaging ICG and the spectral overlap permits visualization of IRDye800 labeled probes (24,25).

Time course studies showed a tumor-specific signal present as early as 6 hours. However, there was non-specific background noise present over the liver, bladder and the skin that decreased over time as the tumor signal increased. Despite the background noise, there was an adequate tumor to background signal at all time points evaluated. A nonspecific background signal over the skin and soft tissue is likely attributable to the circulating probe not yet bound to the tumor or eliminated. This skin and soft tissue signal is minor and will not pose an issue for this construct in clinical surgical use. The signal over the bladder is likely due to a degree of proteolytic degradation and renal elimination of resulting peptides and fluorophore. As most patients undergoing major oncologic surgery are catheterized, this will not likely be an issue in the operative field. However there is significant accumulation of the antibody-dye conjugate at the liver beyond expected for known antibody hepatic accumulation (26,27). This liver signal may be due to additional hydrophobicity from LICOR-IRDye800CW. This signal could potentially be an issue, as it can potentially mask CEA-positive hepatic malignancies or CEA positive colorectal liver metastases. Despite the initial fluorescence at the liver, the signal rapidly decreases after 12 hours and returns close to baseline near 48 hours and beyond. The addition of polyethylene glycol (PEG) groups or attachments of the fluorophore in a conformation that may mask the hydrophobic moieties, could be approaches to improve this issue which will be addressed in future work (28).

Conclusion

Humanized anti-CEA-800 tumor-specific dye specifically labeled orthotopically implanted pancreatic cancer xenografts. The dye successfully co-localized with GFP tagged tumor cells. The longer wavelength allowed for deeper tissue penetration, particularly in areas of the tumor covered by normal pancreatic parenchyma. Humanized anti-CEA antibody conjugated to a radiolabeling agent is undergoing Phase I/II trials. IRDye800CW has also been in a number of Phase I/II trials for FGS of head and neck cancers. Humanized anti-CEA conjugated with the IR-800 dye is a promising agent for future clinical FGS applications.

Acknowledgments

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Synopsis

A humanized anti-CEA antibody conjugated to a near-infrared fluorophore (LICOR-IRDye800CW) brightly labeled orthotopic pancreatic xenografts in situ. The fluorophore-conjugated antibody is a promising construct for future use in clinical surgical navigation.

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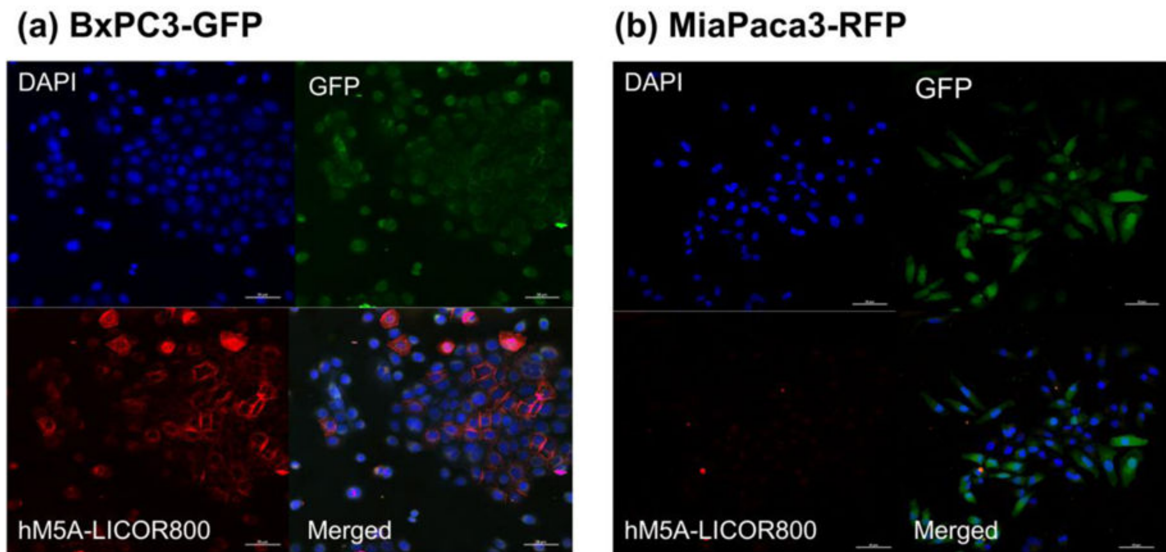

Figure 1. hM5A-IR800 binding to BxPC3-GFP human pancreatic cancer cell line

Staining with hM5A-IR800 showed that the fluorescent antibody bound the CEA-expressing BxPC-3-GFP human pancreatic cancer cell line (Figure 1a). Merged images of the GFP and hM5A-IR800 channels show the fluorescent-antibody localized at cell surfaces. Staining with hM5A-IR800 showed little to no fluorescent antibody binding to low CEA expressing MiaPACA-2-RFP human pancreatic cancer cells (Figure 1b). Merged images of the RFP and hM5A-LICOR800 channel show poor antibody cell localization. MiaPaCa-2-RFP cells were imaged at the RFP channel but represented in green pseudocolor rather than a red pseudocolor for figure consistency.

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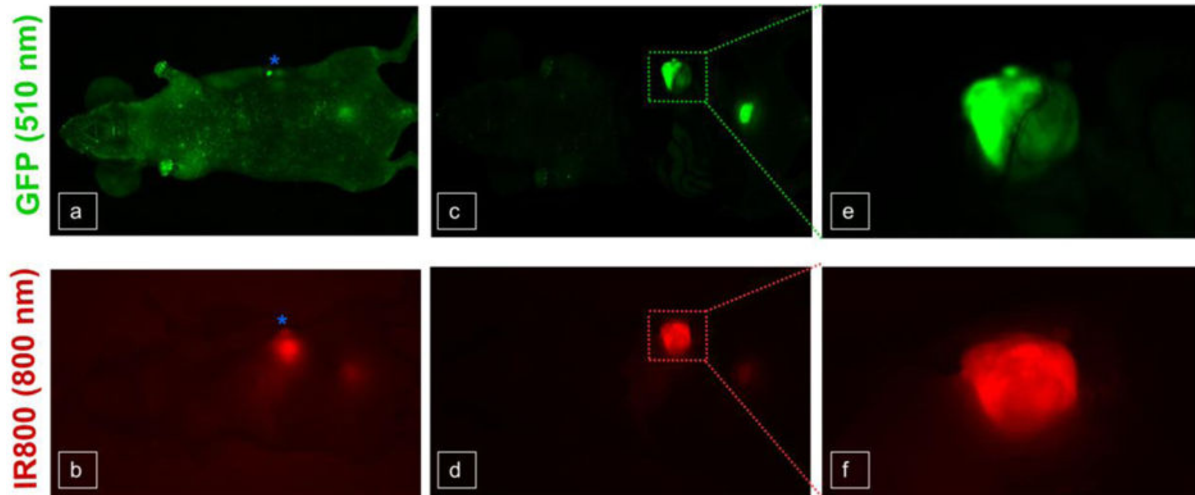

Figure 2. Selective Tumor Labeling of Pancreatic Cancer by hM5A-IR800

Non-invasive imaging of mice at 48 hours showed a detectable fluorescence signal (Figure 2a, 2b). Compared to GFP, the near-infrared wavelength demonstrates a stronger tumor signal through the tissue layers in non-invasive imaging (Figure 2a, 2b) as well as the laparotomy view (Figure 2c, 2d). The areas labeled by hM5A-IR800 clearly overlap with the GFP tagged tumor. Magnified views show signal dampening due to overlying parenchyma and vasculature in the GFP channel (Figure 2e, 2f). The NIR wavelength showed superior tissue penetration.

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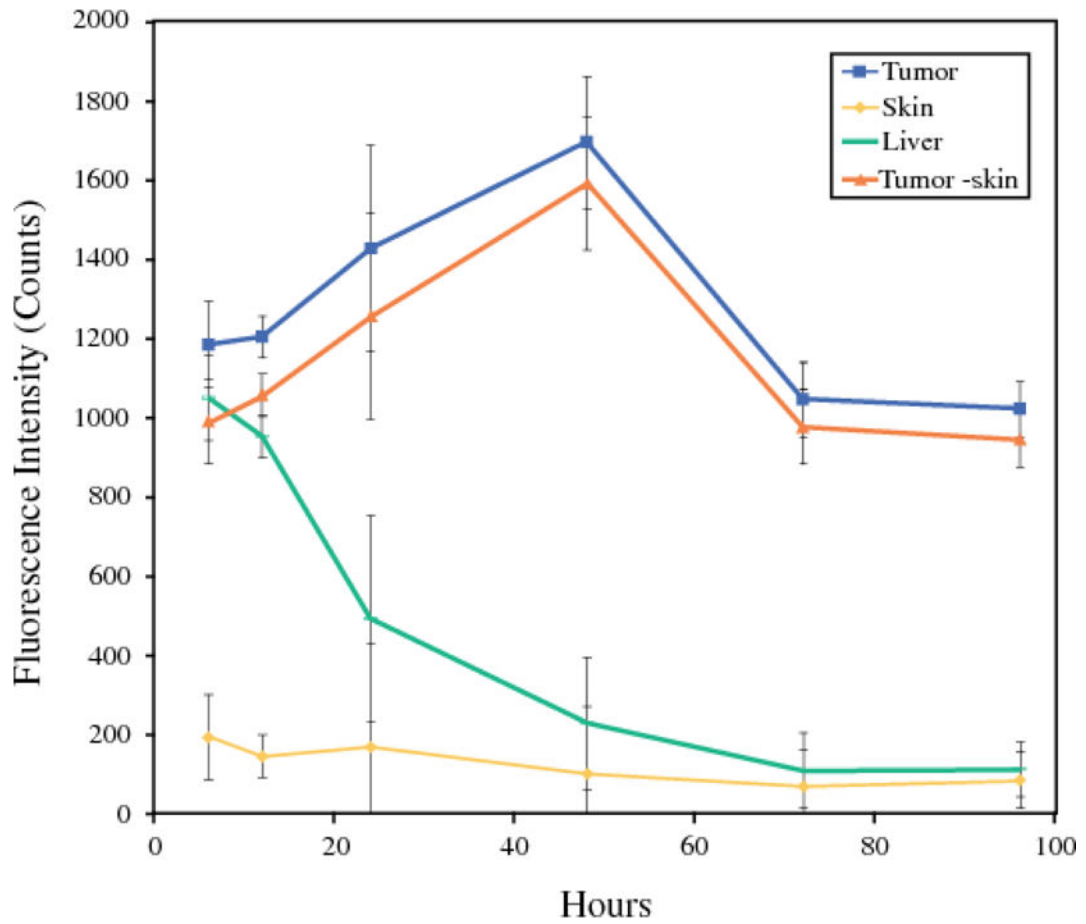

Figure 3. hM5A-IR800 Time course of Fluorescence Intensity

Fluorescence intensity of hM5A-IR800 at 6, 12, 24, 48, 72, and 96 hours after injection. Tumor (red line, square markers), skin (blue line, diamond markers), liver (green line, downward arrowhead markers), and tumor signal adjusted for skin background noise (yellow line, upward arrowhead markers) are represented.

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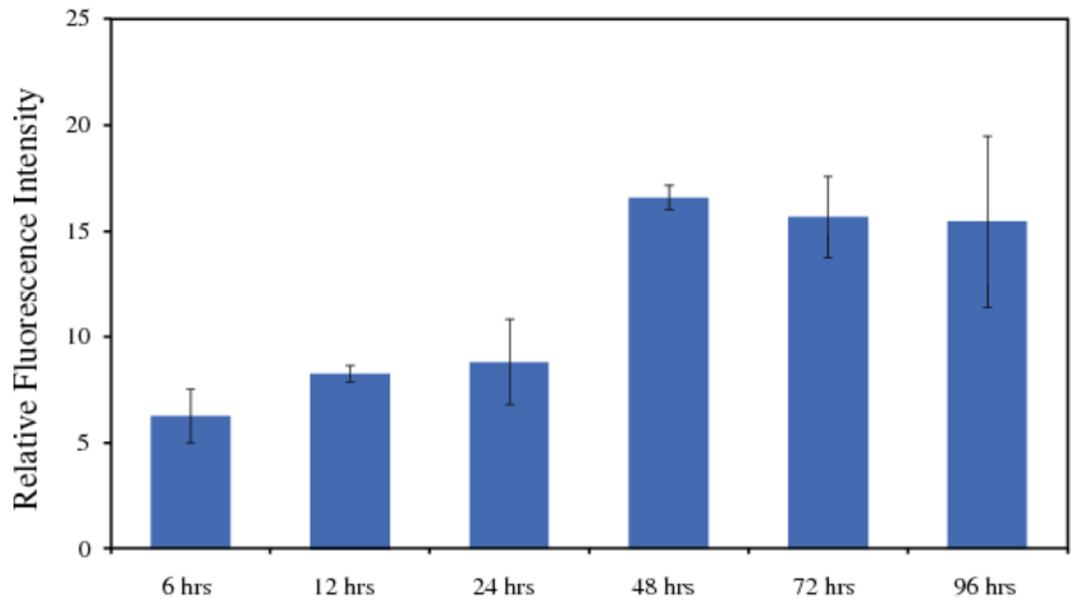

Figure 4. M5A-IR800 Time course of Tumor to Background Ratio

Tumor to background ratio of M5A-IR800 at 6, 12, 24, 48, 72, and 96 hours after injection. The time of peak signal intensity correlated with the maximal tumor-to-background ratio at 48 hours (TBR=16.6).

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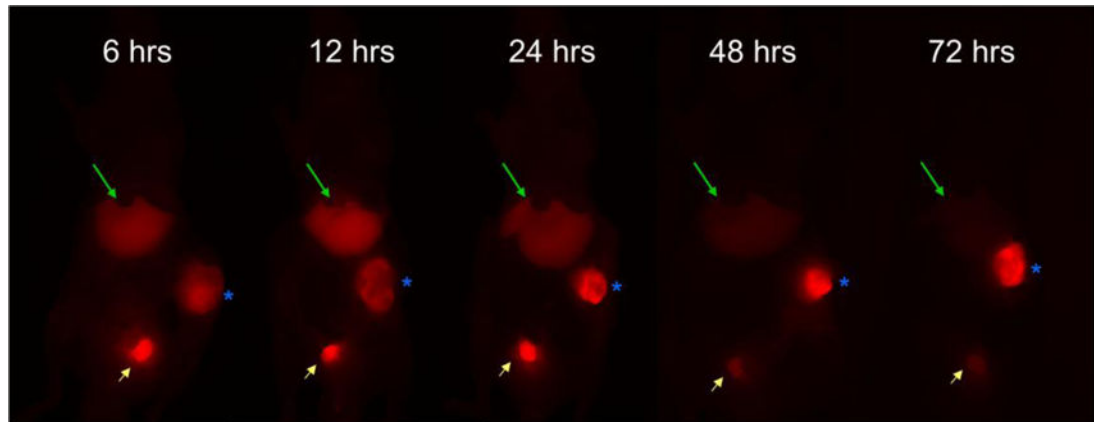

Figure 5. M5A-IR800 Time course

Serial intra-vital images using M5A-IR800 at 6, 12, 24, 48 and 72 hrs. All images demonstrate a fluorescent signal at the tumor at the IR800 wavelength (blue asterisks). The images show additional background noise at the liver (green arrows) and bladder (yellow arrowhead) that steadily decreases over time.