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Pre-clinical imaging of epidermal growth factor receptor with ABY-029 in soft-tissue sarcoma for fluorescence-guided surgery and tumor detection

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Abstract

Background and objectives: Fluorescence-guided surgery using epidermal growth factor receptor (EGFR) targeting has been performed successfully in clinical trials using a variety of fluorescent agents. We investigate ABY-029 (anti-EGFR Affibody® molecule labeled with IRDye 800CW) compared to a small-molecule perfusion agent, IRDye 700DX carboxylate, in a panel of soft-tissue sarcomas with varying levels of EGFR expression and vascularization.

Methods: Five xenograft soft-tissue sarcoma cell lines were implanted into nude mice. ABY-029 and IRDye 700DX were each administered at 4.98 µM. Fluorescence from *in vivo* and *ex vivo* (fresh and formalin fixed) fixed tissues were compared. The performance of three fluorescence imaging systems were assessed for *ex vivo* tissues.

Results: ABY-029 is retained longer within tumor tissue and achieves higher tumor-to-background ratios both *in vivo* and *ex vivo* than IRDye 700DX. ABY-029 fluorescence is less susceptible to formalin fixation than IRDye 700DX, but both agents have disproportional signal loss in a variety of tissues. The Pearl Impulse provides the highest contrast-to-noise ratio, but all systems have individual advantages.

Conclusions: ABY-029 demonstrates promise to assist in wide local excision of soft tissue sarcomas. Further clinical evaluation of *in situ* or freshly excised *ex vivo* tissues using fluorescence imaging systems is warranted.

Keywords

Affibody® molecule; IRDye 800CW; IRDye 700DX; Pearl Impulse; Solaris; Odyssey CLx

Introduction

Fluorescence-guided surgery (FGS) is a rapidly growing field. 5-aminolevulinic acid/ protoporphyrin IX was recently approved for clinical use, and numerous protein targeted and activatable fluorophores are in pre-clinical and Phase 0/1 clinical trials [1–10]. We have developed and begun testing ABY-029, an IRDye 800CW labeled anti-epidermal growth factor receptor (EGFR) Affibody[®] molecule. Affibody[®] molecules are small, engineered peptides that can be optimized and adapted for various imaging applications and therapy [11]. ABY-029 is approved as an exploratory investigational new drug (eIND application 122681) by the US Federal Drug Administration (FDA) and is currently being tested for FGS in Phase 0 studies of several cancers at microdose administration levels [1]. The main advantage of ABY-029 as compared to larger antibody-based targeting agents are that ABY-029 has a significantly shorter plasma half-life measured in minutes to several hours as compared to days, which would allow for administration and surgery to occur on the same day [1,2,12–16]. In addition, ABY-029 is not anticipated to cause any pharmacological response, given that no immunogenic side-effects were observed during toxicity testing using doses equivalent to one-thousand times the human microdose level [1]. However, the tissue distribution and clearance of ABY-029 in solid tumors compared to normal tissues is not well defined.

The application of FGS to orthopaedic surgery has received comparably less attention than in other surgical disciplines. However, several recent pre-clinical and clinical studies have been reported demonstrating the usefulness of FGS for margin detection in soft-tissue sarcomas [17–23]. Soft-tissue sarcomas are mesenchyme-derived cancers composed of malignant cells derived from muscle, fat, tendon, and other connective tissue that are ideally removed in a wide local excision (WLE). WLE involves removal of the tumor, tumor pseudo-capsule, and the surrounding reactive zone, which is known to contain tumor cells. Most surgeons therefore aim to remove an additional 1–2 cm of normal tissue (tumor margin) beyond the pseudo-capsule. Following dissection and analysis by a trained pathologist, margins are graded as positive or negative, determined by whether or not the tumor is exposed at a specimen's surface, respectively. WLE of soft-tissue sarcomas presents several challenges including localizing the tumor within its investing tissues and guiding the surgical dissection around the tumor at a uniform distance from the pseudo-capsule. These challenges have resulted in a positive margin rate of 22–34%, decreasing post-operative survival and increasing tumor recurrence [24–27]. Development of molecular-targeted FGS for WLE of soft-tissue sarcomas may increase the sensitivity and specificity of margin detection and improve outcomes after surgical resection.

ABY-029 is a good candidate for molecular targeted FGS in soft-tissue sarcomas as EGFR is overexpressed in 43–78% of tumors [28–30]. In this investigation we compare ABY-029, a targeted fluorescent reporter, to IRDye 700DX carboxylate, a small-molecule, non-targeted, perfusion agent, in a panel of human sarcoma tumor lines with varying EGFR expression and vascularity. Our overarching goals were to: 1) determine the post-administration time interval that provides optimal tumor-to-background fluorescence ratio; 2) identify the post-resection imaging regimen that provides the most accurate visualization of fluorophore in sarcoma tissue; and 3) compare the performance of three fluorescence imaging systems that

could be, or are currently, used for intraoperative or postoperative image analysis. The information gathered in this pre-clinical mouse model will be used to inform the Phase 0 studies of ABY-029 in human soft tissue sarcomas that are currently ongoing at our institution.

Materials and Methods

Cell lines and culture methods:

All cell lines were purchased from the ATCC (Manassas, VA, USA). Cell lines included: MES-SA (uterine sarcoma), SK-LMS-1 (leiomyosarcoma), SK-NEP-1 (kidney sarcoma, derived from metastatic site, pleural effusion), SW-982 (synovial sarcoma), and VA-ES-BJ (epithelioid sarcoma). All cells were cultured according to ATCC specifications with the exception of SK-NEP-1, which grew only one tumor after several inoculation attempts. Therefore, the SK-NEP-1 tumor was harvested and minced using sterile scissors and digested with 4 mg/mL collagenase for 1 hour at 37°C [31]. The resulting digested tissue was washed with sterile PBS and pushed through a 70 µm nylon cell strainer (BD Falcon). Cells were washed and plated on tissue culture treated plates in RPMI 1640 with 20% Fetal Bovine Serum and 1% v/v penicillin:streptomycin.

Imaging Agents:

The production of Good Laboratory Practices (GLP) ABY-029 has been described previously [1]. Stock solutions of 0.42 mg/mL were obtained from University Alabama at Birmingham (UAB) Vector Production Facility and were further diluted in sterile phosphate buffered saline (PBS) to a concentration of 4.98 µM for experimental administration. IRDye 700DX NHS ester was purchased from LI-COR Biosciences, Inc. (Lincoln, NE). As per instructions from manufacturer, the IRDye 700DX NHS ester was solubilized at 200 µM in PBS at pH 8.5 and left at room temperature for >5 hours under gentle stirring to convert the NHS ester form to IRDye 700DX carboxylate.

Tumor implantation:

All animals were used according to the approved Institutional Animal Care and Use Committee (IACUC) at Dartmouth College. NSG mice (male and female) were purchased from a colony maintained in the Center for Comparative Medicine and Research at Dartmouth College. Tumors were implanted subcutaneously at a density of 1×10^6 cells in 200 µL in 1:1 mixture of Matrigel and culture medium into the left and right flank of each mouse. Three mice, with a total of six tumors, were used per cell line and time point such that a total of 90 mice provided 90 tumors for *in vivo* imaging (one per mouse) and 180 tumors for *ex vivo* imaging (two per mouse). Tumor take rate was 100%, and tumors were used for imaging when they reached ~1,500 mm³.

Imaging protocols:

Prior to imaging, mice were administered 200 µL of a 1:1 solution of 4.98 µM ABY-029 and IRDye 700DX in PBS via intravascular tail vein injection. The 4.98 µM dose is the mouse equivalent to six times the human microdose using the method of Reagan-Shaw et al. (2008) [32], where a microdose in humans is 3.95 µg/kg (237 µg for a 60 kg individual) and the human equivalent in mice 48.7 µg/kg (1.31 µg for a 27 g mouse). Control mice (referred to

as $t=0$) did not receive an injection of any fluorescent agents in order to monitor relative, endogenous autofluorescence levels in the tissues.

At the appropriate time after administering the imaging agents (1, 2, 4, 8, and 24 hours), the mice were anesthetized (1–2.5% isoflurane, 1L/min O₂) and the skin over one subcutaneous tumor was removed. For “*in vivo*” imaging, a black cloth was placed over the entire body with the exception of the tumor and a small amount of surrounding muscle in order to dampen fluorescence signal from liver and kidneys that would not be present during human surgery. The mouse was placed inside of the Pearl Impulse (LI-COR Biosciences, Inc.) and images were collected in both the 700 and 800 nm channels to monitor the fluorescence of IRDye 700DX and ABY-029, respectively. The mice were then sacrificed, and the tumor excised and sliced into 2–5 slices ~3 mm thick. In addition to tumor, normal muscle, fascia, fat, and sciatic nerve were removed and placed on a glass slide. This excision process was repeated for the second tumor on the opposite side of the mouse. “*Ex vivo*” images of the excised tissues from both sides of the mouse were collected for both ABY-029 and IRDye 700DX carboxylate on the Pearl Impulse, Odyssey CLx (LI-COR Biosciences, Inc.) and Solaris (Perkin Elmer, Waltham, MA). The imaging parameters for each imaging system is compared in *Supplemental Data* Table S1.

In vivo imaging data is missing for SK-NEP-1 at the 24-hour time point due to instrument complications. This experimental condition was not repeated in order to minimize use of vertebrate animals, considering that *ex vivo* tissue collection, imaging and pathology were all successful.

Effect of Formalin Fixation on Fluorescence:

Two tumor lines, VA-ES-BJ and MES-SA, were selected as moderate and low EGFR expressing tumors, respectively, and were implanted subcutaneously in both the left and right flanks as described previously. ABY-029 and IRDye 700DX carboxylate (4.98 μ M each) were co-administered to mice ($n = 6$, VA-ES-BJ; $n = 5$, MES-SA) in 200 μ L via tail vein injection 3 hours prior to sacrifice and excision of tumor, muscle, skin, and fat. Control mice ($n = 2$ per tumor line) were not injected in order to evaluate the effect of tissue autofluorescence. Fresh tissue was imaged on the Odyssey CLx and then placed in 10% buffered formalin (Biochemical Science, Inc.) in histological cassettes. At each time interval, the tissues were imaged on the Odyssey CLx and then returned to formalin.

Pathology:

After imaging was complete, all tumor tissue was fixed in 10% buffered formalin (Biochemical Science, Inc.) in histological cassettes. Tissue handling and histopathological staining was performed by the institutional Pathology Translational Research Program as described previously [33]. Standard H&E and EGFR immunohistochemistry (IHC) staining was performed on all tumor tissues. All tissue sections were scanned on the Vectra 3 (Perkin Elmer).

Image Analysis:

All image analysis was performed using FIJI (FIJI Is Just ImageJ) [34–36]. Fluorescence values for each tumor section, muscle, fat, fascia, and sciatic nerve samples were measured individually and grouped by Tumor (all tumor sections) and Normal Tissues (muscle, fat, fascia and sciatic nerve). Tumor-to-Background Ratios (TBR) were calculated by dividing the mean fluorescence in the Tumor sections by the mean fluorescence in the Normal Tissues. Instrument performance was compared by calculating the Contrast-to-Variance Ratio (CVR) defined as:

$$CVR = \frac{\mu(I_{Tum}^{Fl}) - \mu(I_{Norm}^{Fl})}{\sqrt{\sigma_{Tum}^2 + \sigma_{Norm}^2}} \quad \text{Equation 1:}$$

where $\mu(I_{Tum}^{Fl})$ and $\mu(I_{Norm}^{Fl})$ are the average fluorescence intensities in the tumor and normal tissue, respectively, and σ_{Tum} and σ_{Norm} are the standard deviation of the tumor and normal fluorescence, respectively. EGFR IHC staining analysis was performed by using the H DAB Colour Deconvolution script in FIJI to isolate the brown EGFR stain, and then normalizing to the placenta staining positive control slide for each batch of tissues processed to control for staining variations.

Statistical Analysis:

For *in vivo* and *ex vivo* TBR, a two-tailed, equal variance Student's t-test, with $p < 0.05$ was used to determine statistical significance. All time points were compared to the autofluorescence control ($t = 0$ hours) for both ABY-029 and IRDye 700DX data. In addition, for ABY-029 only, the 4- and 8-hour time points were compared to the 24-hour time point to determine clinical relevance.

Results

ABY-029 provides maximal fluorescence intensity within a clinically-relevant timeframe

All tumor lines demonstrated uptake of both the non-targeted IRDye 700DX and the EGFR-targeted ABY-029 to varying degrees (Figure 1). For *in vivo* tumors (Figures 2), the fluorescence intensity of IRDye 700DX generally peaked at 1 to 2 hours, whereas the fluorescence intensity of ABY-029 peaked later at 4 to 8 hours. The normal tissue mainly consisted of muscle but did include fascia surrounding the muscle and potentially sciatic nerve at depth. The normal muscle displayed maximal IRDye 700DX fluorescence at 1 hour with comparable intensity values to that of the tumor tissue. Conversely, ABY-029 fluorescence in the muscle peaked at 4 hours, but was approximately half of the intensity observed in the majority of tumors, with the exception of low EGFR expressing MES-SA.

In the *ex vivo* tissue samples (Figure 3), the maximal IRDye 700DX fluorescence was observed at 1 hour for all sarcoma tumors and normal tissues. In comparison, the *ex vivo* tumor samples displayed maximal ABY-029 fluorescence intensity at 4 to 8 hours. In general, the average fluorescence intensity in the xenograft tumors, followed EGFR

expression (Supplemental Data, Figure S1), with the exception of SK-NEP-1, which demonstrated higher fluorescence than expected. The maximal ABY-029 fluorescence in normal tissues ranged from 1–8 hours, whereas several tumor lines demonstrated a bi-modal peak with maximum fluorescence values at 1 and 4–8 hours. In normal *ex vivo* tissues, ABY-029 fluorescence was substantially lower than tumor at all time points.

Tissues that were formalin fixed generally displayed an initial increase in fluorescence signal over the first 0.5 to 1 h but overall displayed a decrease in fluorescence after 1 h. The change in fluorescence signal over time is summarized in Figure 4, and the percent fluorescence remaining in each tissue after 24 h is summarized in Supplemental Data Table S2. ABY-029 fluorescence was retained at a higher percentage in all tissues with the exception of MES-SA tumor and the fat within the MES-SA mice as compared to IRDye 700DX.

ABY-029 provides significant tumor-to-background ratio in a clinically-relevant timeframe

TBRs for all tumor lines were not statistically different from autofluorescence controls ($t = 0$ h) both at 1 and 2 hours post-ABY-029 administration ($p > 0.05$). The TBRs for all five tumor lines were significantly greater than the autofluorescence control at 4 hours.

Additionally, significant differences to autofluorescence was also observed in SK-LMS-1, SW-982, and MES-SA at 8 and 24 hours, and SK-NEP-1 only at 8 hours (24-hour time point data was missing). VA-ES-BJ did not show significant differences from autofluorescence at either the 8- or 24-hour time points. For *in vivo* IRDye 700DX, SK-NEP-1 at 8 hours was the only TBR significantly different from autofluorescence.

In contrast, the majority of TBRs in *ex vivo* tissues were significantly greater than the autofluorescence control for both ABY-029 and IRDye 700DX (Figure 5). The exception to this was MES-SA, which had very high autofluorescence in the 700-nm channel, and at no time point was significantly different from the pre-injection TBR. In general, the TBRs for *ex vivo* tissues were higher than those of *in vivo* tissues, especially notable at early time points that were non-significant *in vivo*.

In general, the average *in vivo* and *ex vivo* ABY-029 TBR for all tumor lines was highest at 24 hours (Figure 5). The exceptions to this were *in vivo* SK-NEP-1 that was missing this time point and *ex vivo* VA-ES-BJ that showed a declining TBR between 4–24 hours. *In vivo* SW-982 showed statistical significance between the 24-hour time point and the 4- or 8-hour time points ($p = 0.002$ and $p = 0.004$, respectively). All other *in vivo* tumor lines were not deemed different between either the 4- and 8-hour time points and the 24-hour time point. For all *ex vivo* tumor lines there was no statistical significance between the 4- and 8-hour time points with the 24-hour time point, with the exception of the SW-982 24- and 4-hour time point ($p = 0.008$).

Figure 3 displays a representative example of H&E, immunohistochemistry (IHC), vascular distribution (lectin), and fluorescent ABY-029 and IRDye 700DX images for each tumor type. The fluorescence intensity observed in the tumor tissues will be a complex mixture of vascular delivery (lectin fluorescence) and EGFR binding.

Formalin fixation had a varying effect on the TBRs between tumor and the normal tissues as shown in Figure 4. For both ABY-029 and IRDye 700DX, VA-ES-BJ tumors displayed an increase in TBR with 24 h formalin fixation when compared to muscle and fat but had negligible change in TBR when compared to skin. ABY-029 in MES-SA tumors demonstrated a decrease in TBR as compared to muscle and skin and an increase when compared to fat. In MES-SA tumors, the TBR of IRDye 700DX for all normal tissue types was increased with fixation.

Contrast-to-variability ratio (CVR) varied by instrument

Three imaging systems were compared for *ex vivo* sarcoma and normal tissues analyses (Figure 6 and Supplemental Data Table S1). It is evident from the fluorescent images (Figure 6A) that the image detail decreases from Odyssey to Pearl to Solaris and is related to the optical set-up of each instrument. For all instruments, the 800-nm channel had a higher CVR than the 700-nm channel, and the CVR was maximized between 4–24 hours (Figure 6B). The Pearl had the highest CVR for both channels, with the Solaris providing similar, but slightly lower CVRs; while the Odyssey has the lowest CVR.

Discussion

Small molecular weight imaging agents, such as ABY-029, and non-targeted perfusion agents, such as IRDye 700DX, have short plasma half-lives in comparison to larger protein complexes, such as antibodies [16,37]. The short plasma half-life allows agent administration on the same day of surgery, rather than several days prior. However, adequate time must pass between administration and imaging in order to achieve suitable tumor-to-normal tissue background ratios that permit clear distinction of the tumor from surrounding tissue. In this study, it was demonstrated that, regardless of EGFR expression and tumor vascularity, the TBR for all five sarcoma lines increased from 1 hour to 24 hours post-administration. The TBRs of VA-ES-BJ and MES-SA do not increase as substantially as the other tumor lines, likely due to both VA-ES-BJ and MES-SA having high levels of vascularity compared to the other tumor lines (Supplemental Data, Figure S1). ABY-029 produced TBRs that were significantly greater than autofluorescence at 4 hours post-administration in all tumor lines, and negligible statistical differences between 4-, 8- and 24-hour timepoints. This effect was even observed in MES-SA tumors, which express very low levels of EGFR. In comparison, IRDye 700DX does not perform better than autofluorescence. Additionally, upon *ex vivo* observation, it is clear that at 4-hours post-administration ABY-029 fluorescence spatial distribution more closely matches that of EGFR, while IRDye 700DX appears to be collecting in vascular pools and tissue necrotic regions (Figure 4). Therefore, not only does ABY-029 produce clinically relevant TBR values but more accurately represents viable tumor tissue.

Interestingly, from our experience performing a human microdose study of ABY-029 in soft tissue sarcomas, the average tumor excision time was ~4.5 hours post-administration when ABY-029 was administered during pre-operative care (*data not shown*). Here we have shown that ABY-029 fluorescence intensity is maximal between 4–8 hours within all sarcomas and that negligible differences are observed between the TBR in the 4-, 8-, and 24-hour time

points both *in vivo* and *ex vivo*. This suggests that the short plasma life of ABY-029 is suitable for administration for same-day surgery, thus reducing toxicity risk to patients as compared to monoclonal antibodies or second-window indocyanine green [6,21].

Based on our results for soft tissue sarcomas, the highest fluorescence signals were observed *in vivo*, but the difference between tumor and normal tissues was more pronounced in *ex vivo* tissues as demonstrated by higher calculated TBRs than in the *in vivo* conditions. This is likely due to the volume effects of fluorescence. *In vivo* imaging occurs in the whole mouse body where normal tissue surrounding and underneath the tumor can contribute to the overall fluorescence signal, simultaneously dampening the contrast between the tumor and background. This is especially prominent in near-infrared fluorescence where excitation light penetration is likely deeper than the thickness of subcutaneous tumors, which are typically less than 10-mm in thickness in our murine model. In a previous study using soft tissue sarcoma mimicking phantoms, it was determined that the Pearl Impulse could visualize IReDye 800CW at depths substantially over 10-mm when tumor-to-background signals were as high as 3, and over 40-mm when background tissue had no fluorescence background [22]. This has implications for clinical *ex vivo* tissue imaging, where margin assessment may be performed on excised tissues to ensure that tumor fluorescence is adequately far enough from the excised tissue edge. In this case, *ex vivo* analysis can occur at even shorter time points than *in vivo*, although it should be noted that no significant differences were observed 2-hours post-administration in many of the tumors.

Caution must be exercised when using and interpreting formalin-fixed tissue data. Fixation over a short duration can increase fluorescence intensity, whereas long-term fixation (24 hours) causes substantial decreases in fluorescence intensity. The increase in fluorescence intensity at short time periods are likely due to the dehydration and resulting shrinkage of the fresh tissue as it is fixed. This would cause an apparent increase in fluorophore concentration, and subsequent increase in fluorescence intensity as the volume of tissue decreases. After this initial increase, the decline in fluorescence intensity is likely secondary to fluorophore degradation. The effects of formalin fixation were more apparent in IReDye 700DX than in ABY-029, indicating that IReDye 800CW is the more stable fluorophore. Another important factor to consider when analyzing fluorescence uptake in different tissues is that formalin fixation effects vary by tissue type. Therefore, tumor-to-normal background tissue ratios can change disproportionately in relation to fresh tissue, and thus are not accurate for analyzing relative tissue uptake post-fixation.

Contrast-to-variance ratios were calculated for each fluorophore in all three systems tested for *ex vivo* tissue. The Pearl Impulse outperforms the Solaris at time points 4 hours and greater, however under 4 hours the two instruments perform similarly. Although both are wide-field imaging systems, the Pearl Impulse is a closed-box system with laser excitation and the Solaris is an open-air system that utilizes image gating to enable imaging under direct white light [38]. Therefore, although the Pearl Impulse performs the best in terms of CVR, the clinical usefulness of the system is limited because it is not capable of imaging humans during surgery and the fluorescence images do not display the spatial variation required to accurately correlate to pathology. Considering the Solaris can be used with ambient light in a surgically relevant setting, the instrument performs well in comparison to

the Pearl as previously determined [22]. The Odyssey CLx produces high-resolution images with high spatial variation, and although this spatial variability reduces the CVR, it produces images that are most relevant for comparing to pathological images.

Finally, it is important to note that IReDye 700DX is not typically used as a perfusion agent in clinical assessment of vasculature and/or tumors and is not currently approved for clinical use. Indocyanine green (ICG, cardiogreen) and fluorescein are clinically approved by the FDA and are commonly used as vascular perfusion markers in surgery. However, for this study we chose to use IReDye 700DX instead of ICG or fluorescein for several reasons. ICG has considerable spectral overlap with ABY-029 and they cannot be spectrally separated using either the Pearl Impulse or the Odyssey CLx: two instruments commonly used as table top imaging systems for post-excision analysis of tissues during FGS. Fluorescein's maximal fluorescent emission is ~500 nm, which corresponds to a spectral region of high autofluorescence. Although the Solaris is capable of spectrally resolving fluorescein in the 470 nm channel, the Pearl Impulse and the Odyssey CLx are not. We have extensive pre-clinical experience with IReDye 700DX as a perfusion agent [37,39] and used it here in order to recapitulate the biological pharmacokinetics and processes of clinically-relevant agents.

Conclusions

ABY-029 is a small peptide, near-infrared fluorescent reporter that binds EGFR with good signal-to-background ratios and demonstrates promise to assist in WLE of soft tissue sarcomas with administration on the day of surgery. ABY-029 provides useful tumor-to-normal background tissue contrast *in vivo* and *ex vivo* from 4 to 24 hours after administration. High-resolution visualization of *ex vivo* ABY-029 fluorescence demonstrates spatial distribution similar to EGFR expression, whereas this was not observed with IReDye 700DX perfusion dye. Lastly, we determined that the Solaris imaging system is suitable for clinical imaging, while the Odyssey CLx is exceptional at imaging tissues for correlation to pathology. ABY-029 is a promising imaging agent for FGS, and further clinical evaluation with fluorescence imaging systems is warranted.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Synopsis:

The ability to accurately detect soft-tissue sarcomas with varying levels of epidermal growth factor receptor expression using a molecular targeted Affibody[®] molecule is compared to that of a non-specific perfusion agent. Clinically relevant imaging ratios, fluorophore susceptibility to formalin fixation, and imaging system detection sensitivity are compared.

Figure 1. Fluorescence intensity of ABY-029 (*solid lines*) and IRDye 700DX (*dashed lines*) over 24 hours in various soft tissue sarcoma xenograft models and normal tissues. The displayed images are for the Pearl imaging system. Similar trends were observed for the Solaris and Odyssey imaging systems (not shown).

Figure 2.

In vivo soft tissue sarcoma tumors display varying levels of ABY-029 and IRDye 700DX uptake at 4 hours post-administration. Images were collected on the Pearl and are scaled to the same relative intensities to show differences in uptake. The animal was covered with a black drape with a small opening cut into the fabric to exclude high fluorescent signal from abdominal organs. The white dashed line indicates the tumor region, all other visible fluorescence within the field is normal muscle.

Figure 3.

Ex vivo xenograft soft tissue sarcoma tumors demonstrate varying levels of EGFR expression and corresponding uptake of ABY-029 and IRDye 700DX at 4 hours post-administration imaged on the Odyssey scanner. Odyssey scanner images are displayed on the same scale (*right*) for each fluorophore to demonstrate the varying uptake observed between tumor types.

Figure 4. Effects of formalin fixation on ABY-029 (*solid*) and IRDye 700DX (*dashed*) over time. *Top*: Changes in fluorescence intensity from fresh ($t=0$) to 24 hours post-fixation. *Bottom*: The change in tumor-to-background ratio (TBR) from fresh to 24 hours post-formalin fixation.

Figure 5.

Tumor-to-background ratio (TBR) varies over time and tissue type imaged on the Pearl Impulse. *In vivo* measurements (*left column*) demonstrate that all tumors have significantly greater TBR than the autofluorescence values at $t = 0$ h for ABY-029 (*white box*) for at least one time point, but mainly at 4 hours or greater. IRDye 700DX TBR (*grey box*) only showed statistical significance at 8-hours in the SK-NEP-1 tumor line. No *in vivo* TBR showed statistical significance at $t = 2$ h. *Ex vivo* TBR (*right column*) demonstrated significant

differences from the autofluorescence control at many time points for both ABY-029 and IRDye 700DX.

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Figure 6.

Comparing three fluorescence imaging systems using contrast-to-variance ratio (CVR). A) A representative ABY-029 fluorescence image from all three systems is provided, using the 4-hour time point and the 800-nm channel for SK-LMS-1 tumor and normal tissues. B) The Pearl performed the best for both ABY-029 and IRDye 700DX imaging agents, but the Solaris was comparable. Note that the Odyssey displays the most fluorescence detail, such that the large variation in fluorescence intensity observed causes low CVR. The highest CVRs were observed between 4 and 24 hours for all imaging systems.