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A Ratiometric Threshold for Determining Presence of Cancer During Fluorescence-guided Surgery

Jason M Warram, PhD¹, Esther de Boer, BSc¹, Lindsay S. Moore, MD¹, Cecelia E Schmalbach, MD¹, Kirk P Withrow, MD¹, William R Carroll, MD¹, Joshua S Richman, MD, PhD², Anthony B Morlandt, MD, DDS³, Margaret Brandwein-Gensler, MD⁴, and Eben L Rosenthal, MD¹

¹Division of Otolaryngology, University of Alabama at Birmingham, Birmingham, AL, USA, 35233

²Division of General Surgery, Department of Surgery, University of Alabama at Birmingham, Birmingham, AL, USA, 35233

³Department of Oral & Maxillofacial Surgery, University of Alabama at Birmingham, Birmingham, AL, USA, 35233

⁴Division of Anatomic Pathology, Department of Pathology, University of Alabama at Birmingham, Birmingham, AL, USA, 35233

Abstract

Background&Objective—Fluorescence-guided imaging to assist in identification of malignant margins has the potential to dramatically improve oncologic surgery. However a standardized method for quantitative assessment of disease-specific fluorescence has not been investigated. Introduced here is a ratiometric threshold derived from mean fluorescent tissue intensity that can be used to semi-quantitatively delineate tumor from normal tissue.

Methods—Open-field and a closed-field imaging devices were used to quantify fluorescence in punch biopsy tissues sampled from primary tumors collected during a phase 1 trial evaluating the safety of cetuximab-IRDye800 in patients (n=11) undergoing surgical intervention for head and neck cancer. Fluorescence ratios were calculated using mean fluorescence intensity (MFI) from punch biopsy normalized by MFI of patient-matched tissues. Ratios were compared to pathological assessment and a ratiometric threshold was established to predict presence of cancer.

Results—During open-field imaging using an intraoperative device, the threshold for muscle normalized tumor fluorescence was found to be 2.7, which produced a sensitivity of 90.5% and specificity of 78.6% for delineating disease tissue. The skin-normalized threshold generated greater sensitivity (92.9%) and specificity (81.0%).

Conclusion—Successful implementation of a semi-quantitative threshold can provide a scientific methodology for delineating disease from normal tissue during fluorescence-guided resection of cancer.

Corresponding author: Jason M. Warram, Ph.D., Assistant Professor, Department of Surgery, University of Alabama at Birmingham, Volker Hall G082, 1670 University Blvd, Birmingham, AL 35294-0019, Mojack@uab.edu, (205) 996-5009.

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Keywords

standardized imaging; fluorescence-guided surgery; surgical oncology; head & neck cancer

Introduction

Over the past decade, fluorescence-guided imaging to navigate cancer resection has been shown to significantly improve the number of complete resections and progression free survival [1–7]. While this imaging strategy has yielded promising results during initial trials, many elements of the approach must be validated more rigorously before successful clinical application can occur [8,9]. Selective imaging data presentation from clinical cases has been sufficient to show proof of principle, however, clinical implementation will require a reproducible scientific methodology for determining the positivity of cancer using fluorescent-guided techniques. Clinical trials for cancer specific imaging report ambiguous and subjective values for determining the amount of fluorescence contained within tissue during surgical assessment. Typical classifications used to define the extent of fluorescence within imaged tissue include “solid”, “moderate”, or “vague” [3,10–13]. These values will be insufficient to guide surgical care in advanced stage trials and need to be supported by specific criteria for determining positive fluorescence. The bulk of computable data collected for analysis in these clinical trials illustrates the subjective nature of fluorescence-guided imaging where the surgeon forms a subjective assessment in real-time based on the fluorescence intensity observed in the field of view. Data reporting in this manner will be confounded by variations in tumor heterogeneity, differences between surgeons, and time interval between dosing and imaging. Additionally, this non-standardized reporting makes intertrial comparisons challenging, which hampers the long-term advancement of the strategy. While tumor-to-background ratio (TBR) or signal-to-background ratio (SBR) has been used to describe fluorescence intensity in some studies [14–16], the “background” is poorly defined and can include multiple tissue types ranging from muscle, skin, fat, stroma, connective tissue, etc. These ratios are helpful to differentiate the intensity of the fluorescence contrast between patients, but do not provide a specified optical diagnostic value for delineating disease from normal tissue. As this promising modality is widely adopted and enters routine clinical use, tumor detection techniques must advance beyond subjective observation.

To be successful, fluorescence guided optical imaging will need to objectively detect a subclinical volume of tumor embedded within normal tissue. To this end, we propose the use of a ratiometric threshold to objectively diagnose the tumor/normal interface in real-time, when the modality can have the greatest impact on complete resection. Comparison of fluorescence intensity produced from the positive tissue divided by the fluorescence of patient-matched muscle or skin, which serves as a standardized background or negative value. Use of patient derived skin or muscle serves as an internal anatomical control for subsequent imaging of tumor margins, wound bed, and unknown resected tissues during “back-table” imaging. This formula controls for variations between fluorescent uptake in stromal tissue and variations between patients. Once a threshold is established, the ratiometric value can be used to reliably identify subclinical disease embedded within

normal tissue. Additionally, it is hypothesized that the value can be applied universally across patient populations, similar to standardized uptake value (SUV) used during positron emission tomography.

We develop a repeatable ratiometric threshold for detection of small fragments (<2 mm) within normal tissue using patient-derived tissues from a recent clinical trial (ClinicalTrials.gov Identifier: NCT01987375) evaluating the safety of cetuximab-IRDye800 in patients undergoing surgical intervention for squamous cell carcinoma (SCC) arising in the head and neck. Imaging was performed on fresh tissue using an intraoperative fluorescence-imaging device (Luna, Novadaq, Canada), a closed-field fluorescence-imaging device for back-table assessment (Pearl, Li-Cor, Lincoln Nebraska), and slide-mounted sections using a fluorescence scanner (Odyssey, Li-Cor) for pathological evaluation. The presence of cancer in tissues was confirmed during histological analysis by a board-certified pathologist, which served as the gold standard.

Methods

Tissue collection

Punch biopsies (4mm) of tumor, surrounding tissue, muscle, and skin were collected by the ablative surgeon from 11 consented patients enrolled in a dose escalation (2.5 mg/m², 25 mg/m², and 62.5 mg/m²) clinical trial (Clinicaltrials.gov Identifier: NCT01987375) evaluating the safety and tumor-specificity of systemically injected cetuximab-IRDye800 for surgical navigation in patients with SCC. For cohort 1 (2.5mg/m² dose), 14 punch biopsies were collected (9 malignant, 5 normal) from three patients with tumors originating from the lateral tongue and floor of mouth. For cohort 2 (25mg/m² dose), 71 punch biopsies were collected (32 malignant, 39 normal) from five patients with tumors originating from floor of mouth, lateral tongue, and neck metastasis. For cohort 3 (62.5mg/m² dose), 16 punch biopsies were collected (10 malignant, 6 normal) from three patients with tumors originating from the floor of mouth and septum. All patients were diagnosed with conventional SCC. Per trial design, patients received respective infusion of cetuximab-IRDye800 3–4 days prior to scheduled surgical procedure. Informed consent was obtained from all individual participants included in the study. All patient data were anonymized and all experiments using the specimens were conducted in accordance with the rules and regulations approved by the University of Alabama Institutional Review Board.

Fluorescence imaging and analysis

Imaging of collected punch biopsies and normal tissue was performed using an FDA-approved open-field fluorescence imaging device (LUNA, Novadaq, Ontario, Canada) and a closed-field fluorescence device (Pearl Impulse, LI-COR, Lincoln, NE). Samples were imaged in the operating room and in surgical pathology within 1 hour of tissue resection. Using integrated instrument software (LUNA: SPY-Q, Pearl: Image Studio), areas of high fluorescence were quantified with custom regions of interest and mean fluorescence intensity (MFI) were recorded. Similarly, MFI was determined for skin and muscle samples. From these MFI values, a ratio was calculated by dividing MFI of punch biopsy tissue by MFI of patient-matched muscle or skin. The ratiometric values were recorded for each

punch biopsy sampled and correlated with pathological determination (gold standard) performed by a board-certified pathologist.

Histology

After imaging, punch biopsy tissue was formalin fixed and embedded in paraffin. Multiple sections (5 μ m) were obtained and a board-certified pathologist performed histological analysis using haematoxylin and eosin (H&E) staining. Punch biopsies were determined positive for presence of cancer (denoted as tumor) or negative for presence of cancer (denoted as normal). Adjacent unstained sections were imaged using a fluorescence scanner (Odyssey, LI-COR) specifically optimized for IRDye800.

Statistics

A receiver operator characteristic (ROC) curve was generated to determine the diagnostic performance of the ratiometric threshold in both devices and normalizing tissue types. ROC curves were estimated for fold-increase in fluorescence separately for reference tissue type (skin and muscle) and for instrumentation (open-field and closed-field) systems using the package pROC in R version 3.1.1. The area under the curve (AUC) was estimated with bootstrapped 95% confidence intervals and DeLong's test was used to compare the results between tissue types for each device.

Results

Collection and imaging of punch biopsy tissue

In order to determine the optimum diagnostic threshold for predicting the presence of cancer using fluorescence-guided navigation, the resected primary tumor was sampled using punch biopsy tissue obtained from random intratumoral and adjacent areas, as determined by the ablative surgeon. As shown in Figure 1, punch biopsy (4mm) tissue was obtained from a primary tumor mass with 2cm+ margins (Figure 1a) resected from a patient in the 25mg/m² dose group. After punch biopsy samples were collected, the primary specimen was fluorescently imaged (Figure 1b) using the open-field device prior to pathological processing. Figure 1c shows open-field and closed-field fluorescent imaging of resected punch biopsy tissue and corresponding pathological determination. Roman numeral annotations denote origin of respective punch biopsy sample.

Open-field fluorescent imaging

Open-field near-infrared (NIR) fluorescent imaging was performed on punch biopsy tissues to determine a diagnostic threshold specific to the imaging device. For comparison, both muscle and skin MFI were used as the respective ratiometric denominator to determine an independent threshold specific to the normalizing factor. Figure 2a shows the dose-specific distribution of ratiometric values for tumor-positive and normal punch biopsy tissue (determined using gold standard) normalized by muscle for the 25mg/m² dose group (32 tumor, 39 normal) and the 62.5mg/m² dose group (10 tumor, 6 normal). Comparatively, Figure 2b shows the ratiometric values for the respective tissue when MFI of patient-matched skin is used as the denominator. Each data point represents the ratiometric value (punch biopsy tissue MFI / patient-matched muscle MFI) calculated for each punch biopsy

sampled. When imaging using the open-field intraoperative device, a sensitivity of 90.5%, specificity of 78.6%, positive predictive value of 80.9%, and negative predictive threshold of 89.2% was calculated using the optimal ratiometric threshold of 2.7 against muscle (Table 1). When using the MFI of patient-matched skin to generate the ratio, a sensitivity of 92.9%, specificity of 81.0%, positive predictive value of 83.0%, and negative predictive threshold of 91.9% was determined when using the optimal ratiometric threshold of 1.1 (Table 1). Representative brightfield and open-field fluorescence images are shown for tumor (Figure 2c) and normal (Figure 2d) punch biopsy tissues for the 25mg/m² dose group and 62.5mg/m² dose group (Figure 2e–f, respectively). For the open-field device, fluorescence was not detected in tissues sampled from the microdose (2.5mg/m²) group.

Closed-field fluorescent imaging

In Figure 3a, the distribution of ratiometric values calculated for each tissue punch biopsy is shown for the 2.5mg/m², 25mg/m², and 62.5mg/m² dose groups when patient-matched muscle is used as the normalizing factor during closed-field device imaging. As shown in Table 1, a sensitivity of 92.0%, specificity of 74.5%, positive predictive value of 79.3%, and negative predictive value of 89.7% was determined at the optimal ratiometric threshold of 3.2 for the muscle-normalized ratios. In Figure 3b, distribution of ratiometric values are shown for the respective dose groups when MFI of patient-matched skin is used as the denominator. When using a skin-normalized, optimal ratiometric threshold of 1.5, a sensitivity of 92.0%, specificity of 83.0%, positive predictive value of 85.2%, and negative predictive value of 90.7% was determined when imaging with the closed-field device. Representative brightfield and fluorescence images are shown of tumor-positive and normal punch biopsy tissues for the 2.5mg/m² (Figure 3c–d), 25mg/m² (Figure 3e–f), and 62.5mg/m² (Figure 3g–h) dose groups.

Histological localization of fluorescence

To determine the cancer-specific nature of the observed fluorescence, cancer-containing areas were co-localized with areas of high fluorescence using H&E staining and fluorescence images acquired from adjacent unstained sections using a fluorescence scanner (Odyssey, LI-COR). Representative H&E (Figure 4a) and fluorescence (Figure 4b) images are shown from a cancer-containing punch biopsy obtained from the 25mg/m² dose group. Zoomed inset areas demonstrate the cancer-specific fluorescence observed for punch biopsies containing tumor tissue.

Receiver operator characteristic

To statistically evaluate the performance of the ratiometric threshold to differentiate tumor from normal tissue, a receiver operator characteristic curve was generated for the open-field (Figure 5a) and closed-field (Figure 5b) imaging devices with empirical 95% confidence intervals for the AUCs, each generated from 2000 bootstrapped replications to summarize expected variability in the AUC. Analysis was performed using both skin and muscle as ratiometric denominators to assess the power of these tissues to serve as normalizing factors for cetuximab-IRDye800 imaging. For the open-field device, the AUC for skin-normalized tissue was 0.842 (0.691–0.994) while muscle-normalized tissue was 0.835 (0.689–0.981). When ratiometric values were plotted for the closed-field device, the AUC for skin-

normalized tissue was 0.895 (0.832–0.958) while muscle-normalized tissue was 0.840 (0.757–0.923). For each of the ROC curves, there was a significantly ($P<0.01$) greater AUC than the chance diagonal with a 95% confidence interval.

Discussion

Fluorescent guided-surgery using cancer specific contrast agents remain a unique opportunity to significantly improve outcomes in the management of patients with solid malignancies. This is the first study to apply a rigorous scientific model to assist in detection of tumor tissue using fluorescence imaging. We demonstrate that the use of a ratiometric threshold after systemic injection of cetuximab-IRDye800 can be used for successfully delineating tumor from normal tissue. While this proof of principle study was performed in SCC of the head & neck, this approach may be applied to reliably determine the presence of malignancy in any tumor type during real-time assessment of unknown tissues that are normalized for inter-patient and intra-tumoral variances. To achieve this broad application in tumor types of any anatomical location, the patient-specific fluorescence of an internal anatomical control (muscle or skin) is used to account for these differences. During open-field fluorescence imaging with an intraoperative imaging device, the threshold for muscle normalized tumor fluorescence was found to be 2.7. This threshold was shown to be sensitive (90.5%) for delineating disease tissue, however high background in skin occurring at the 62.5mg/m² dose confounded the ratio thereby reducing specificity (78.6%). When a skin-normalized threshold was used to standardize fluorescence in punch biopsy tissues, the sensitivity (92.9%) and specificity (81.0%) improved. For the closed-field device, sensitivity and specificity was relatively similar to the open-field device for muscle-normalized (92.0% and 74.5%, respectively) and skin-normalized (92.0% and 83.0%, respectively) punch biopsy tissues when a threshold of 3.2 was used for muscle-normalized tissue and 1.5 for skin-normalized tissue. These results showed that the ratiometric threshold varied slightly between normalizing tissues and imaging devices. This suggests that agent and device-specific thresholds need to be uniquely determined as the combinations of agent and device are approved and introduced into patient use. When determining the appropriate normalizing tissues, probe pharmacokinetics and offsite targets need to be considered in order to provide an unbiased, tumor-specific threshold for detection. In the current study, skin and muscle were compared to account for inherent properties of cetuximab to target EGFR in normal skin, which affected the specificity at the higher dose.

During fluorescence-guided surgery, a fluorescence contrast agent is administered to provide real-time definition between disease and normal tissue during intraoperative imaging. The strategy was initially evaluated for resection of malignant glioma using orally administered 5-aminolevulinic (5-ALA) to identify borders of malignant disease [10]. During a phase 3 trial, fluorescence-guided resection using 5-ALA produced a significantly greater number of complete resections compared to white-light resection [7]. The approach has been expanded to include other cancer types in combination with various fluorescent probes. Examples of probes being evaluated in patients include: indocyanine green (ICG) for sentinel lymph node detection [1,2], fluorescein (FITC) labeled peptides for ovarian cancer resection [3], and methylene blue for fluorescence-guided resection of breast [6] and cervical cancer [4,5].

It is clear that the use of fluorescence imaging to guide surgical navigation is advancing towards approved use in multiple cancer types. However, there is a great need for widespread standardization of multiple components associated with this technique before routine clinical use can be fully implemented. To this end, a recent assembly was held by a fluorescence-guided surgery study group during the 2013 European Molecular Imaging Meeting to identify obstacles in the advancement of this modality [8]. Specific issues discussed during the meeting included regulatory approval for trial design, imaging device standardization, quality control for agent manufacture, and the requirement for good clinical and scientific practice. Among issues critical in the advancement of the technique is to incorporate a scientific methodology for delineating disease from normal tissue in order to circumvent the subjective nature of optical imaging. Here, we introduce a strategy to overcome these limitations.

For the current study, two NIR fluorescence-imaging devices were used to image and quantify levels of fluorescence in the punch biopsy tissues. The open-field device can be used in the operative field to localize fluorescence in real-time with malignant margins in the wound bed. The closed-field device, which is specifically optimized for the NIR IRDye800 molecule used in the study, was utilized in a “back-table” setting with resected tissues. As the utility of fluorescence-guidance advances, it is not clear which setting will provide the greatest benefit. It is envisioned that the intraoperative device will be used to localize tumor for resection and wound bed scanning in a post-resection setting to ensure no residual cancer remains. The “back-table” device affords greater sensitive detection of microscopic disease due to the low background associated with a black-box system and can be utilized to detect residual cancer during margin analysis or to assist the pathologist in localizing areas for histological assessment during frozen section analysis. These two devices can work in concert to provide a synergistic benefit leading to cancer-free resection. When the ratiometric threshold approach is incorporated, the color look-up table threshold can be adjusted to permit tumor-specific scanning where the ratio is gated to color only those areas greater than the pre-determined ratio threshold. In addition, the ratiometric formula can act as a standardized fluorescence value, similar to standardized uptake value used in positron emission tomography, for comparison between patients and institutions.

One limitation of the current study is the small sample size and absence of a validation group. This small proof of principle study cannot expect to provide thresholds and measures of discrimination that can be generalized with confidence to a broader population. However, this study does provide an estimate of the method’s potential to discriminate between normal tissue and tumor thereby introducing a desperately needed tool for standardization during fluorescence-guided surgery. The values reported here were compiled using optimal ratios from our specific data set. Ultimately, determining thresholds for general use will require a larger and more general sample.

Here, a ratiometric threshold was introduced to provide a method for objective assessment of tumor fluorescence during fluorescence-guided surgery. This formula produced a highly sensitive, instrument-specific threshold for predicting presence of cancer in the punch biopsy tissues tested. ROC analysis revealed the ratiometric threshold approach to be a powerful diagnostic tool. While the method was shown to be highly sensitive, artifacts

inherent to EGFR binding in the skin confounded the specificity. However, considering the consequences of a false negative during cancer localization in the surgical setting, the optimal threshold should be leveraged towards the greatest sensitivity to confidently identify true positive tissues.

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References

1. Troyan SL, Kianzad V, Gibbs-Strauss SL, et al. The FLARE intraoperative near-infrared fluorescence imaging system: a first-in-human clinical trial in breast cancer sentinel lymph node mapping. *Ann Surg Oncol*. 2009; 16:2943–2952. [PubMed: 19582506]
2. Hirche C, Dresel S, Krempien R, Hunerbein M. Sentinel node biopsy by indocyanine green retention fluorescence detection for inguinal lymph node staging of anal cancer: preliminary experience. *Ann Surg Oncol*. 2010; 17:2357–2362. [PubMed: 20217256]
3. van Dam GM, Themelis G, Crane LM, et al. Intraoperative tumor-specific fluorescence imaging in ovarian cancer by folate receptor- α targeting: first in-human results. *Nat Med*. 2011; 17:1315–1319. [PubMed: 21926976]
4. van der Vorst JR, Hutteman M, Gaarenstroom KN, et al. Optimization of near-infrared fluorescent sentinel lymph node mapping in cervical cancer patients. *Int J Gynecol Cancer*. 2011; 21:1472–1478. [PubMed: 22027751]
5. Schaafsma BE, van der Vorst JR, Gaarenstroom KN, et al. Randomized comparison of near-infrared fluorescence lymphatic tracers for sentinel lymph node mapping of cervical cancer. *Gynecol Oncol*. 2012; 127:126–130. [PubMed: 22796548]
6. Tummers QR, Verbeek FP, Schaafsma BE, et al. Real-time intraoperative detection of breast cancer using near-infrared fluorescence imaging and Methylene Blue. *Eur J Surg Oncol*. 2014; 40:850–858. [PubMed: 24862545]
7. Stummer W, Pichlmeier U, Meinel T, et al. Fluorescence-guided surgery with 5-aminolevulinic acid for resection of malignant glioma: a randomised controlled multicentre phase III trial. *Lancet Oncol*. 2006; 7:392–401. [PubMed: 16648043]
8. Snoeks TJ, van Driel PB, Keereweer S, et al. Towards a successful clinical implementation of fluorescence-guided surgery. *Mol Imaging Biol*. 2014; 16:147–151. [PubMed: 24249642]
9. Rosenthal EL, Zinn KR. Putting numbers to fluorescent guided surgery. *Mol Imaging Biol*. 2013; 15:647–648. [PubMed: 23836503]
10. Stummer W, Novotny A, Stepp H, et al. Fluorescence-guided resection of glioblastoma multiforme by using 5-aminolevulinic acid-induced porphyrins: a prospective study in 52 consecutive patients. *J Neurosurg*. 2000; 93:1003–1013. [PubMed: 11117842]
11. Adam C, Salomon G, Walther S, et al. Photodynamic Diagnosis Using 5-Aminolevulinic Acid for the Detection of Positive Surgical Margins during Radical Prostatectomy in Patients with Carcinoma of the Prostate: A Multicentre, Prospective, Phase 2 Trial of a Diagnostic Procedure. *Eur Urol*. 2009; 55:1281–1288. [PubMed: 19328622]
12. Fukuhara H, Inoue K, Satake H, et al. Photodynamic diagnosis of positive margin during radical prostatectomy: Preliminary experience with 5-aminolevulinic acid. *Int J Urol*. 2011; 18:585–591. [PubMed: 21658132]

13. Millesi M, Kiesel B, Woehrer A, et al. Analysis of 5-aminolevulinic acid-induced fluorescence in 55 different spinal tumors. *Neurosurg Focus*. 2014; 36:E11. [PubMed: 24484249]
14. Gleysteen JP, Newman JR, Chhieng D, et al. Fluorescent labeled anti-EGFR antibody for identification of regional and distant metastasis in a preclinical xenograft model. *Head Neck*. 2008; 30:782–789. [PubMed: 18228526]
15. Day KE, Beck LN, Heath CH, et al. Identification of the optimal therapeutic antibody for fluorescent imaging of cutaneous squamous cell carcinoma. *Cancer Biol Ther*. 2013; 14:271–277. [PubMed: 23298904]
16. Sorace AG, Saini R, Rosenthal E, et al. Optical fluorescent imaging to monitor temporal effects of microbubble-mediated ultrasound therapy. *IEEE Trans Ultrason Ferroelectr Freq Control*. 2013; 60:281–289. [PubMed: 23357902]

Synopsis

The use of fluorescence to guide surgical resection of cancer has been introduced to the clinic, however there is currently no clear method of identifying tumor in real-time using this method. We introduce a ratiometric threshold for objective assessment of tissue fluorescence during fluorescence-guided surgery. This formula produced a highly sensitive methodology for predicting presence of cancer in the tissues tested.

Figure 1. Imaging of tissue-derived punch biopsies
(a) Resected specimen from 25mg/m² dose group containing primary tumor with wide surgical margin is shown with (b) imaging on back-table using open-field imaging system. Annotated areas denote origin of punch biopsies I–IV. (c) open-field and closed-field fluorescent imaging of punch biopsy is shown with corresponding pathology status as determined by histological evaluation. Colorimetric threshold was fixed between images.

Figure 2. Open-field fluorescent imaging of punch biopsy tissue

Using an intraoperative fluorescent imaging device, the ratio of mean fluorescent intensity (MFI) for pathology-confirmed tumor or normal tissue over the MFI of (a) patient-matched muscle or (b) patient-matched skin is shown for the 25mg/m² and 62.5mg/m² dose groups. Representative white-light and fluorescent images are shown for (c) tumor 25mg/m² dose, (d) normal 25mg/m² dose, (e) tumor 62.5mg/m² dose, and (f) normal 62.5mg/m² dose groups. Each data point represents MFI ratio for a single punch biopsy. Colorimetric threshold was fixed between images.

Figure 3. Closed-field fluorescent imaging of punch biopsy tissue

Using a closed-field fluorescent imaging device, the ratio of mean fluorescent intensity (MFI) for pathology-confirmed tumor or normal tissue over the MFI of (a) patient-matched muscle or (b) patient-matched skin is shown for the 2.5mg/m², 25mg/m², and 62.5mg/m² dose groups. White-light and fluorescent images acquired using closed-field system are shown for (c) tumor 2.5mg/m² dose, (d) normal 2.5mg/m² dose, (e) tumor 25mg/m² dose, (f) normal 25mg/m² dose groups, (g) tumor 62.5mg/m² dose, and (h) normal 62.5mg/m² dose. Colorimetric threshold was fixed between images.

Figure 4. Fluorescence localization in tumor-containing tissue

(a) Representative H&E stain of tumor-containing punch biopsy from the 25mg/m² dose group is shown with (b) corresponding fluorescence scan of adjacent section.

Figure 5. Receiver operating characteristic analysis

(a) A receiver operating characteristic curve was generated for the open-field imaging device using mean fluorescence intensity ratios determined using skin (AUC: 0.887, $P < 0.01$) and muscle (AUC: 0.875, $P < 0.01$). (b) Analysis was also performed for the closed-field imaging device using skin (AUC: 0.895, $P < 0.01$) and muscle (AUC: 0.840, $P < 0.01$).

Table 1

Accuracy of disease detection among devices and ratios tested.

Device	Standardizing tissue	Ratiometric threshold	Sensitivity	Specificity	Positive predictive threshold	Negative predictive threshold
Open-field	Muscle	2.7	90.5%	78.6%	80.9%	89.2%
	Skin	1.1	92.9%	81.0%	83.0%	91.9%
Closed-field	Muscle	3.2	92.0%	74.5%	79.3%	89.7%
	Skin	1.5	92.0%	83.0%	85.2%	90.7%