Intraoperative Near-Infrared Imaging of Surgical Wounds after Tumor Resections Can Detect Residual Disease

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Abstract

Purpose: Surgical resection remains the most effective therapy for solid tumors worldwide. The most important prognostic indicator for cure following cancer surgery is a complete resection with no residual disease. However, intraoperative detection of retained cancer cells after surgery is challenging, and residual disease continues to be the most common cause of local failure. We hypothesized that visual enhancement of tumors using near-infrared imaging could potentially identify tumor deposits in the wound after resection.

Experimental Design: A small animal model of surgery and retained disease was developed. Residual tumor deposits in the wound were targeted using an U.S. Food and Drug Administration–approved imaging agent, indocyanine green, by the enhanced permeability and retention effect. A novel handheld spectrometer was used to optically visualize retained disease after surgery.

Results: We found residual disease using near-infrared imaging during surgery that was not visible to the naked eye or micro-CT. Furthermore, examination of tumor nodules was remarkably precise in delineating margins from normal surrounding tissues. This approach was most successful for tumors with increased neovasculature.

Conclusions: The results suggest that near-infrared examination of the surgical wound after curative resection can potentially enable the surgeon to locate residual disease. The data in this study is the basis of an ongoing Phase I/II clinical trial in patients who undergo resection for lung and breast cancer. Clin Cancer Res; 18(20); 5741–51. ©2012 AACR.

Introduction

Cancer remains a major health problem and affects more than 1.4 million Americans each year (1). Surgical resection is the most effective therapy for early stage solid tumors, and more than 50% of patients with cancer undergo surgery with curative intent each year (2). A complete resection at the time of surgery is the single most important factor in predicting long-term survival and improves the cure rate by 4- to 11-fold for many cancers (2). Unfortunately, up to 40% of patients leave the operating room with deposits of cancer cells left behind at the time of surgery (2).

Patients with recurrent disease tend to have poor outcomes (2). Local recurrences occur from cancer cells found at the edge of the resection field, or the "surgical margin." Local recurrences are a known risk of surgery, thus radiation therapy is often given postoperatively to prevent relapses. For example, in breast cancer, after the standard-of-care lumpectomy to remove a breast nodule, adjuvant radiation is usually administered to the surgical field (3). However, radiation has significant side effects and morbidity such as scarring, collateral tissue damage, and patient fatigue.

Surgeons have a limited ability to locate residual tumor cells at the surgical margin. For the last 2 centuries, surgeons have used their hands and their eyes to locate and evaluate these margins, but this approach has limitations. Frozen sections are typically carried out during surgery to examine margins, and representative samples of the tumor margin are sent to the pathologist for rapid examination to determine if tumor cells are present. However, studies have shown that it would take 3,000, 6 μm thick sections to fully evaluate the margins of a 2 cm breast biopsy, an impossible task intraoperatively (4, 5).

Our group and others (6–8) have been investigating the use of real-time imaging during surgery to examine tumor margins and residual neoplasia. Near-infrared (NIR) imaging is a safe, low-energy (10⁻¹ eV) approach to imaging and delivers 10⁻⁸ less energy than a routine chest radiograph (10⁻⁴ eV) making it safe for the surgeon, patient, and surgical team. Indocyanine green (ICG) is an innocuous, U.S. Food
In the United States, more than 700,000 people undergo cancer surgery each year for curative intent. Up to 40% of those patients develop local recurrences within 5 years of their initial operation, likely because of missed cancer deposits. Our group has developed a novel approach to image for residual cancer during surgery to confirm disease clearance. This approach uses a safe, nontoxic, nonradioactive strategy using indocyanine green, an U.S. Food and Drug Administration–approved near-infrared fluorophore. Multiple cancer-type mouse models were used to show its utility. These data are currently being used to move forward with a Phase I/II clinical trial for patients with breast and lung cancer. This unique tactic of intraoperative imaging with near-infrared techniques will likely impact all fields of surgical oncology within the next decade.

**Translational Relevance**

In the United States, more than 700,000 people undergo cancer surgery each year for curative intent. Up to 40% of those patients develop local recurrences within 5 years of their initial operation, likely because of missed cancer deposits. Our group has developed a novel approach to image for residual cancer during surgery to confirm disease clearance. This approach uses a safe, nontoxic, nonradioactive strategy using indocyanine green, an U.S. Food and Drug Administration–approved near-infrared fluorophore. Multiple cancer-type mouse models were used to show its utility. These data are currently being used to move forward with a Phase I/II clinical trial for patients with breast and lung cancer. This unique tactic of intraoperative imaging with near-infrared techniques will likely impact all fields of surgical oncology within the next decade.

and Drug Administration (FDA)–approved imaging dye that can be injected into patients and be detected by NIR imaging. ICG imaging is not possible for most diagnostic applications because of the lack of tissue penetration of the emitted light through the skin (9), however, when the body cavity is open, NIR imaging devices can detect ICG at depths of 5 to 10 mm in tissue.

ICG is avidly taken up in solid tumors that have "leaky capillaries" due to the enhanced permeability and retention (EPR) effect (10). The EPR effect is a property by which small molecules (i.e., nanoparticles) accumulate in tumors because of the presence of defective endothelial cells and wide fenestrations that characterize neovascularization in cancer tissues (11). Although ICG is not tumor specific, for purposes of intraoperative diagnostic imaging, the identification of any abnormal tissues is more important than specificity for cancer deposits. We hypothesized that NIR imaging could be used to detect tumor margins and discover residual tumor deposits during surgery. Because ICG is preferentially retained in tumors, we used a novel local recurrence model in small animals to test our NIR imaging platform. We found that residual tumor deposits could be detected with remarkable accuracy. There was no associated toxicity and surgical outcomes were remarkably improved by combining NIR imaging with standard-of-care surgical resection. These data are the basis of an ongoing clinical trial in lung cancer and breast cancer.

**Materials and Methods**

**Mouse studies**

Female C57BL/6 (B6, Thy1.2), BALB/c, athymic Ncr-nu/nu, and B6-129/J1 hybrid mice were purchased from Charles River Laboratories and Jackson Laboratories. All mice were maintained in pathogen-free conditions and used for experiments at ages 8 weeks or older. The Animal Care and Use Committees of the Children’s Hospital of Philadelphia, The Wistar Institute, and the University of Pennsylvania approved all protocols in compliance with the Guide for the Care and Use of Laboratory Animals.

**Cell lines**

The murine malignant mesothelioma cell line, AB12, was derived from an asbestos-induced tumor and has been previously described in detail (12). The murine esophageal squamous carcinoma cell line, AKR, was derived from mouse esophageal squamous epithelia with cyclin D1 overexpression via Epstein–Barr virus Ed-L2 promoter in p53 deficient genetic backgrounds (13). The murine lung cancer cell line, TC1, was derived from mouse lung epithelial cells immortalized with HPV-16 E6 and E7 and transformed with the c-Ha-ras oncogene (14). The spontaneously metastatic murine lung cancer line, LKR, was derived from an explanted pulmonary tumor from an activated KrasG12D mutant mouse that had been induced in an F1 hybrid of 129SvJ and C57BL/6 (14). The metastatic non-small cell lung cancer (NSCLC) cell line, murine Lewis lung carcinoma (LLC), was obtained from American Type Culture Collection. AE17 is an asbestos-derived murine mesothelioma cell line (kindly provided by Steven Albelda, University of Pennsylvania).

AB12, LKR, AKR, and LLC cell lines were cultured and maintained in high-glucose Dulbecco’s Modified Eagle’s Medium (Mediatech) supplemented with 10% FBS (Georgia Biotechnology), 1% penicillin/streptomycin, and 1% glutamine. TC1 and AE17 cell lines were cultured in RPMI (RPMI 1640 Medium, Mediatech) 10% FBS, 1% penicillin/streptomycin, and 1% glutamine. Cell lines were regularly tested and maintained negative for Mycoplasma spp.

**GFP transduction of murine cancer cells in vitro**

AE17 tumor cells were infected with the lentiviral vector pELNS bearing the EF1α promoter to develop GFP fluorescence. The packing of the plasmid into the lentivirus has been previously described (15). Labeled cells were confirmed to express GFP by flow cytometry and fluorescent microscopy.

**Reagents**

Pharmaceutical grade ICG was purchased from Akorn, Inc. (IC-GREEN, NDC 17478-701-02). Animals were dosed with 7.5 mg/kg of ICG via intravenous injection 24 hours before imaging.

**Animal flank tumor models**

Mice were injected subcutaneously on the flank with $5 \times 10^5$ AKR tumor cells (C57Bl/6 mice), $1 \times 10^6$ AB12 cells (BALB/c mice), $1.5 \times 10^6$ TC1 cells (C57Bl/6 mice), $2 \times 10^6$ LLC cells (C57Bl/6 mice), or $2 \times 10^6$ LKR cells (Bl/6/J11) unless otherwise noted. Tumor cells for subcutaneous injections were suspended in 100 μL PBS. Tumor volume was calculated using the formula $(3.14 \times \text{long-axis}^2 \times \text{short-axis}^2)/6$.

Surgery was carried out on mice bearing flank tumors using an established partial resection model (16). Surgery was carried out when tumors reached approximately 800
mm³. Mice were anesthetized with intramuscular ketamine (80 mg/kg) and xylazine (10 mg/kg), shaved, and the surgical field sterilized before surgery. A 1 to 2 cm incision was made adjacent to the tumor and 80% of the tumor was removed using standard blunt dissection technique. After imaging, the incision was closed using sterile silk 4-0 sutures. Buprenorphine (0.2 mg/kg) was administered at the time of surgery and 6 hours postoperatively to provide analgesia. Preoperative treatment was unknown to the investigator conducting surgery and making tumor measurements.

**Canine tumor model**

Three canines with spontaneously occurring soft tissue sarcomas were recruited from the University of Pennsylvania School of Veterinary Medicine. All protocols were approved by the Animal Care and Use Committees of the University of Pennsylvania and were in compliance with the Guide for the Care and Use of Laboratory Animals. Animals underwent ICG injection and soft tissue sarcomas in canines were resected by standard surgical practice. All tumors and surrounding tissues were imaged in vivo and ex vivo.

**NIR and fluorescent imaging**

The Li-Cor Pearl Impulse (LI-COR Biosciences) was used to visualize ICG present within the tissues of the animal. The IVIS Lumina II (Caliper Life Sciences) was implemented to visualize GFP fluorescence expressed by the AE17-GFP cell line. Both machines are housed at the University of Pennsylvania’s Small Animal Imaging Facility (Philadelphia, PA).

The handheld NIR imaging system has been previously described in detail (17). In brief, a Raman Probe detector was incorporated into a cylindrical stainless steel sampling head integrated with a 5 m, 2-fiber cable; 1 for laser excitation and the other for light collection. The sampling head and fiber cable were coupled via a fiber cable connector to a spectrometer. The combined sampling head and spectrometer system has a wavelength range of 800 to 930 nm with 0.6 nm spectral resolution for NIR fluorescence measurement. The excitation light was provided by a 785 nm, 100 mW continuous-wave diode laser.

**Micro-CT scanning**

To show the size of residual disease that remains after surgery, micro-CT was conducted before surgery and following surgery and wound closure (MicroCAT II, ImTeK, Inc.). A series of 10 representative animals underwent partial or total resection of their flank tumors, and then imaged using the MicroCAT II (18).

**Immunohistochemical stains**

Animals were euthanized at designated intervals. Their tumors were harvested and bisected with one-half either placed in Tissue-Tek OCT and stored at −80°C or in formalin for paraffin sectioning. To detect endothelial cells, monoclonal CD31 (mAB390; 19) was raised from hybridoma supernatant and purified. Frozen tumor sections were prepared as previously described (20). CD31 expression was quantified by counting the number of positively staining cells in 4 high-powered (×400) fields (21). Five slides for each specimen were analyzed as previously described.

**Statistical analysis**

For flow cytometry, immunohistochemistry, flank tumor volume studies comparing differences between 2 groups, we used unpaired Student t tests. For studies comparing more than 2 groups, ANOVA with appropriate post hoc testing was implemented. Kaplan–Meier curves were used to determine postoperative median survival. Postoperative survivals (defined as the time from surgery to the time which flank tumor volume reached 1,500 mm³) for treatment groups were compared using the log-rank statistic. Differences were considered significant when P < 0.05. Data are presented as mean (SE), unless otherwise noted.

**Results**

**A surgical model for residual tumor after surgery develops local relapses**

To model the human scenario for local recurrences after surgery, several flank xenografts were established in C57Bl/6 and BALB/c mice. We tested NSCLC (TC1, LLC, LKR), mesothelioma (AB12), breast cancer (4T1), esophageal (AKR), and melanoma (B16) cell lines to confirm the broad generalizability of this model. More than 7 experiments, syngeneic immunocompetent mice (n = 150) were injected with tumor cells into the right flanks. In 3 weeks, animals developed well-encapsulated flank tumors that could be visualized and palpated (Fig. 1A). Once the tumors reached a mean volume of 800 mm³, animals underwent surgery with positive margins (n = 120) or complete resection (n = 30). Two independent observers were then asked to distinguish which animals had complete resection versus residual disease. They were instructed to locate any tumor deposits while the wound was open. They did not have prior knowledge about which animals underwent partial versus complete resection. The observers independently examined the wound after all bleeding had ceased but without any enhanced visual tools such as surgical loupes (Fig. 1A). The observers recorded which animals had suspicious areas. After recording their decisions following visual inspection, they were then allowed to palpate the wound.

If at least 1 of the 2 observers felt there was a residual nodule, the animal was labeled as an incomplete resection. Manual palpation added little to the study. None of the residual tumors were able to be palpated by either the observer or the surgeon. The underlying pelvis or rib cage often made it difficult to distinguish bony protruberances from the actual disease. The observers were able to detect only 11 of the 120 residual tumors and falsely assigned 2 of the 30 animals with complete resection with residual tumors. Visual inspection of the wound for residual disease thus had a sensitivity of 9.2%,
Within 30 days, the local recurrences reached a large size (>800 mm³). When the tumors measured 800 mm³, they were either partially resected with positive margins or completely removed. In the partial resection group, the surgeon used a #15 scalpel to sharply divide the tumor and leave the smallest possible residual nodule (<50%) that was technically feasible (ranged 2–4 mm in diameter). This tumor deposit was typically left attached to either the skin or underlying muscle.

Animals were allowed to recover and then monitored for recurrence. Greater than 97% of all animals with partial resection developed local recurrences that could be visualized within 1 week (Fig. 1B). Local recurrences rapidly developed potentially because of several factors that have been previously described, such as preestablished tumor microenvironment, perioperative immunosuppression, and the presence of wound-related growth factors (14, 16). Within 30 days, the local recurrences reached a large size (>800 mm³) and the animals were euthanized (Fig. 1B).

Residual tumor in the surgical bed fluoresces after incomplete resection

To determine if the NIR imaging platform could detect residual tumor deposits after surgery, animals with large established tumors were resected and the surgical bed was examined for retained disease by (i) visual inspection; (ii) Li-Cor Pearl Impulse; and (iii) the portable handheld NIR device. Syngeneic mice (n = 60) were injected with TC1 tumor cells into the right flank (not over the liver) and developed large tumors (>800 mm³). When the tumors measured 800 mm³, they were either partially resected with positive margins or completely removed. In the partial resection group, the surgeon used a #15 scalpel to sharply divide the tumor and leave the smallest possible residual nodule (<5%) that was technically feasible (ranged 2–4 mm in diameter). This tumor deposit was typically left attached to either the skin or underlying muscle. A, a representative animal from each group is shown. Two independent observers first visually inspected the wound, and then palpated the wound to determine which animals had residual disease versus curative surgery. Animals (n = 20) without obvious tumor to independent observers underwent micro-CT scans to determine if residual disease could be radiographically located. White arrows designate flank tumors. B, following surgical intervention, all animals with (i) positive margins and (ii) total resections were monitored for recurrence of their flank tumors. Tumor volume versus time and Kaplan–Meier survival curves for a typical flank TC1 (n = 20) experiment are depicted.
If an area of high uptake was discovered, the tissues surrounding the suspicious region were imaged at 2 mm intervals from the focus in 4 perpendicular directions. NIR imaging revealed 46 animals (85%) had residual nodules. The NIR fluorescence was significantly higher in these residual nodules [mean fluorescence 41,226 ± 1,429 arbitrary units (a.u.)] compared with the average of the surrounding background fat, skin, muscle, and fascial tissues. The fold difference of the center of the residual nodule was based on the background value. On average, tumors were 16.9-fold more fluorescent than the surrounding tissue (Fig. 2B).

Within 4 mm from the closest margin, there was residual fluorescence that was 3- to 5-fold higher than the background. The small size of the residual nodule did not technically permit accurate correlation of residual tumor size and fluorescent signal intensity.

In vivo, imaging on the Li-Cor Pearl Impulse was not as sensitive as the handheld device in detecting NIR fluorescence. In 56% (n = 30) of the cases, the Li-Cor Pearl Impulse did not detect any residual disease despite high measurements of fluorescence from the NIR handheld device. The handheld NIR device did not detect residual nodules in 8

Figure 2. Residual disease in the surgical bed fluoresces after incomplete resection. C57bL/6 mice were injected with 1.5e6 TC1 cells into the right flank. Once tumors reached 800 mm³, animals were intravenously injected with 7.5 mg/kg ICG. Twenty-four hours later, the surgeon sharply removed 95% of the tumor with minimal residual disease. Two independent observers were then designated to locate suspicious margins. If residual disease was detected, the animal was eliminated from the study. The remaining animals had their surgical bed imaged by the Li-Cor Pearl Impulse. The surgical field was then arbitrarily assigned a virtual grid and 3 readings using the NIR handheld machine were recorded and averaged in each sector. The mean background value was calculated from this data. If a region of high fluorescence was detected, serial readings were conducted circumferentially at 2-mm intervals surrounding the suspicious location. A, a representative C57bl/6 mouse with a flank TC1 is depicted. After surgical resection, the residual nodule could not be visualized by 2 independent observers or with assistance from the Li-Cor Pearl Impulse. The handheld device rapidly detected the lesion and a typical map of the surgical wound is presented. B, the mean fluorescence from the residual nodules (n = 54) and the peritumoral tissue 4 mm from the margin is shown in the bubble graph. The fold difference between the background signal, 4 mm margin, and actual residual nodule are represented in the bar graph. C, duplicate experiments were conducted in mice bearing AE17-GFP tumors to evaluate if ICG expression was occurring in residual tumors versus leaking into surrounding tissues following the surgery.
animals (15%). Eight animals were not deemed to have residual nodules by visual inspection, Li-Cor Pearl Impulse or handheld NIR imaging. The handheld NIR device thus had a sensitivity of 85.2% [95% confidence interval (CI) 0.72–0.93] whereas the Li-Cor Pearl Impulse had a sensitivity of 29.6% (95% CI 0.18–0.44) in discovering residual nodules after surgery.

To confirm that the ICG was specific to tumor cells and not inflammatory tissues that may exist in a surgical wound, we repeated our experiments in a mesothelioma cell line (AE17) labeled with GFP. GFP is expressed ubiquitously by the transformed tumor cells, thus we hypothesized it would be a precise method to compare the margins of tumors with NIR fluorescence. We injected 20 immunodeficient mice with AE17-GFP tumor cells in the flank. Once the tumors reached 800 mm³, they were resected with positive margins. We imaged mice looking before and after surgery using ICG and GFP (Fig. 2C). There was an exact overlay of fluorescent signals when imaged at 820 nm (ICG) and 510 nm (GFP). This confirmed that the tumor tissue was preferentially retaining ICG as compared with the surrounding normal tissue. This characteristic was not altered after surgical resection.

**Residual tumor deposits have elevated fluorescent signals ex vivo**

To confirm the presence of residual disease, the nodules were harvested and analyzed ex vivo. The residual nodules were reimaged using the Li-Cor Pearl Impulse and the handheld NIR machine following resection (Fig. 3A). In several cases, peritumoral tissue such as fat and underlying fascia and muscle was harvested along with removal of the residual nodule. The margins of the nodule could be more easily delineated ex vivo using the handheld device. The lack of background noise from the mouse abdominal cavity and the ability to position the nodule flat on a dry surface improved the fluorescence readings (Fig. 3A). In all cases, an additional piece of tissue distant from the tumor such as muscle, fat, and epidermis was harvested. The fluorescence signal in the residual nodules typically increased in intensity using the handheld device. Frequently, the fluorescence signal from the residual nodule saturated the NIR imaging device. The small residual nodules that could not be detected by the Li-Cor Pearl Impulse in vivo were often visualized ex vivo. The fluorescence increased on average 1- to 2-fold using the NIR imaging device. All tissues were sectioned and underwent hematoxylin and eosin (H&E) staining. All nodules detected by the handheld NIR device were found to have cancer cells (Fig. 3B). The signal intensity from peritumoral tissues (i.e., muscle, fat, skin) at the margins decreased when imaged ex vivo (Fig. 3C).

Next, we attempted to determine the smallest residual nodule that could be detected by the imaging system and the Li-Cor Pearl Impulse. The observers were instructed to measure residual nodules with calipers. There was a poor correlation (P > 0.3) between size measurements of residual nodules between individuals. Because of the small size of the nodules, this procedure was not technically feasible. All the nodules (n = 18) that were under 3 mm in largest diameter (as assessed by the reviewers) were discovered only by the handheld NIR system and not the Li-Cor Pearl Impulse. Our smallest nodule containing residual disease was less than 2 mm. This nodule had a 5.8-fold increase of signal intensity from background tissue. The Li-Cor Pearl Impulse was clearly able to detect any nodule that was more than 5 mm in largest diameter (n = 4).

Together, these data suggest that once a residual nodule is removed from the animal, repeat imaging can be a useful diagnostic test to confirm successful removal. It also permits rapid examination of tumor margins for potentially residual disease, and it provides a valuable resource for surgeons to rapidly determine disease presence in residual tissues without delay in histologic confirmation.

**Intraoperative detection and removal of residual nodules prevents tumor relapses**

To determine if imaging could be used to identify residual nodules that could then be removed at surgery (i.e., the “clinical utility”), we repeated the above procedure in 50 mice. Mice were injected with TC1 tumor cells (n = 25) and AB12 tumor cells (n = 25) into the flank. Once tumors reached 800 mm³, all of the animals were injected intravenously with ICG and the tumors partially resected 24 hours later. An independent team examined all animals by visual inspection alone and eliminated 6 animals that had obvious residual nodules. The remaining animals were then randomized to either imaging by the handheld NIR spectroscopic device (n = 22) versus no further procedures (n = 22). The control animals had their wounds closed, allowed to recover, and were observed. In the other group, we chose an arbitrary 5-fold cutoff (nodule:background signal) for selecting abnormal tissues that were then removed. Twenty of the 22 animals (91%) that were imaged were discovered to have areas of increased IR signal (mean signal 16.9 ± 3.7-fold difference) and these areas were harvested. One of the remaining animals had a suspicious nodule by NIR imaging (3.8-fold higher fluorescence signal) but did not meet the 5-fold cutoff, therefore, the area was not removed. The other animal did not have any uptake that was discernable despite diligent examination. In addition, we placed this animal in the Li-Cor Pearl Impulse and did not discover any residual disease, although there was residual fluorescence in the tail vein, which confirmed that mouse had received intravenous ICG. All nodules that were harvested were prepared for histology. All of the animals then had their wounds closed, were allowed to recover, and were observed over time. None of the animals died perioperatively.

All 22 animals that underwent incomplete surgery without imaging developed local flank recurrences within 1 week. In contrast, only 2 animals in the group that underwent surgery with image-guided resection developed local recurrences within 1 week. The 20 animals that underwent successful discovery of residual nodules did not develop recurrences and were followed for at least 30 days after surgery (Fig. 4A). Imaging also appeared to be highly accurate. In every case of resection, pathologic examination of the resected material showed the presence of tumor
tissue. These data show there was a significant survival advantage to examining the surgical wound for residual disease with the NIR imaging system before closing the wound, with only 2 animals having nodules below the threshold of the handheld device.

**ICG fluorescent signal intensity correlates with microvascular density**

During our experiments in various cell lines (TC1, AB12, AE17, LLC, 4T1, B16), we noted a significant variation in the fluorescence between cell lines. In our animal experiments above, TC1 tumors consistently expressed the highest fluorescence in recurrent tumors (>35,000 a.u.). AB12 tumors, on the other hand, typically had the lowest fluorescence (~15,000 a.u.). AE17 tumors tended to have fluorescence values that were intermediary of TC1 and AB12. The ratio of background fluorescence to that of the tumor increased in the order of AB12, AE17, and TC1. We thus postulated that the difference in fluorescence is attributable to differing levels of vascularization throughout the tumors, which is consistent with the EPR effect (22).

Syngeneic mice underwent injection of TC1, AE17, or AB12 in the right flank. Once tumors reached 800 mm³, they were opened and directly imaged. The tumors were imaged at the center and at 4 locations 90° apart at the periphery. The average of the peripheral measurements was compared with the average of 3 readings from the opposite flank. We then harvested the tumors, sectioned them, and...
Conducted a microvascular density (MVD) assay on TC1, AE17, and AB12 tumors. The assay showed that TC1 tumors have significantly higher vasculature than AB12 tumors (136 vessels/hpf vs. 32, \( P < 0.0001 \)) and AE17 tumors (136 vessels/hpf vs. 62, \( P < 0.0001 \)), thus providing one potential explanation for the difference in fluorescence between tumors (Fig. 4B).

**Canine model of soft tissue sarcoma confirms NIR imaging of tumor margins**

Three canines with soft tissue sarcomas were resected between December 2011 and April 2012 by standard veterinary practice. During the operation, all 3 tumors could be easily palpated and tumor margins were evident by visualization and palpation by the veterinary surgeon (Fig. 5A). In vivo imaging was able to detect all tumor margins without difficulty (Fig. 5A). All tumors had 12- to 15-fold increased fluorescence compared with surrounding tissue. No residual tumor deposits were discovered in the surgical bed after tumor resection. After removing the soft tissue sarcomas, the tumors were analyzed for tumor margins using the handheld NIR imaging device (Fig. 5B). All resection margins were negative for any evidence of cancer deposits. All tumors were ultimately discovered to have negative margins by histopathology. No local recurrences have been detected to date in these 3 canine patients (follow-up 45–110 days).

**Discussion**

Over the last 2 centuries, surgeons have depended on 2 tools in the operating room to detect disease—their eyes to look for suspicious masses and their hands to feel for abnormal tissue (23). Surgeons make subjective decisions based on experience of anatomical tissue planes and tumor spread in deciding where to excise tumors to obtain disease-free margins. However, this is historically inaccurate, especially in complex fields including those with prior radiation, infection, or trauma. For example, at University of Miami, one experienced urologist carried out 100 consecutive radical prostatectomies and recorded intraoperatively if he suspected the tumor margins were positive or negative based on visual clues and palpation (24). Despite his intraoperative assessment that the surgical margins were negative in all 100 patients, the true pathologic margins were positive in 39% of cases. The intraoperative assessment of the margin status had a high false-negative rate and a sensitivity of only 7%. The sensitivity of the intraoperative assessment of tumor location was 73%, and the positive predictive value was 65%. Our results confirm that visual inspection and manual palpation alone are insufficient for discovering residual tumor nodules (Figs. 1, 2, and 4A). This work proposes using NIR imaging techniques, in real-time, during surgery for accurate discovery of residual disease in the surgical wound. This work shows this approach using an FDA-approved fluorophore and a novel imaging device that is compact and handheld.

Several techniques have been developed to assist surgeons improve upon intraoperative decision making. For example, rapid pathologic frozen section can be used to verify the entire tumor has been removed. The pathologist will microscopically study a few limited locations on the specimen to determine if residual tumor cells remain in the patient before the surgeon ends the operation. Although frozen section does improve assessment of surgical margins, it also has limited sensitivity and practicality (25). Routine frozen section analysis is time consuming and can lead to inadequate assessment of tumor margins in large specimens and loss of diagnostic material in small specimens (26). Time-intensive techniques, such as frozen section analysis, also have the disadvantage of prolonging the surgical procedure and thereby the time the patient remains...
anesthetized. To improve accuracy from 90% to 99% for detecting cancer requires the pathologist to triple the number of blocks to be examined (27).

NIR fluorophores are probes that emit at 700 to 850 nm and have significant advantages for imaging because of minimal interfering absorption and fluorescence from biologic samples, inexpensive laser diode excitation, reduced scattering, and enhanced tissue penetration depth (28). To date, only one NIR probe, ICG, has been approved by the FDA. ICG, is a water-soluble, anionic, amphiphilic tricarbocyanine probe with a hydrodynamic diameter of 1.2 nm, and excitation and emission wavelengths in serum at 778 and 830, respectively. ICG has been in clinical use since the 1950s for ophthalmic angiography, determining cardiac output and hepatic function measurements. However, it has only recently shown real practicability and feasibility in the field of surgical oncology (9).

NIR fluorophores such as ICG can be delivered to tumors via a passive targeting mechanism. Nanometer-sized particles accumulate preferentially at tumor sites through an EPR effect (10, 11). In order for tumor cells to grow, they stimulate a complex process of tumor angiogenesis. Tumor cell deposits as small as 200 nm depend on hypervascularization and extensive production of vascular permeability factors to deliver oxygen and nutrients. These vessels are characterized by defective endothelial cells that have wide fenestrations, lack a smooth muscle layer, and have wide lumens. In addition, these tumor deposits lack efficient vascular and lymphatic drainage, which leads to abnormal molecular and fluid transport dynamics (11). As a consequence, small molecules such as ICG can readily leak into the cancer tissues. Once in this acidic tumor microenvironment, ICG will preferentially bind albumin and other tissue proteins that cause it to accumulate over time. In our study, we found a direct correlation with highly vascular murine

Figure 5. A canine with a spontaneous abdominal wall sarcoma underwent image-guided surgery. After ICG injection, (A) a canine abdominal was opened to reveal a well-demarcated soft tissue sarcoma. The primary tumor was imaged in 8 radial directions from the center of the tumor and fluorescence averaged. The tumor bed was imaged before wound closure. B, after resection, ex vivo fluorescence measurements were again conducted.
tumors and ICG fluorescence (Fig. 4B). Human cancers that have been well formed may not have leaky capillaries on the interior, however, we postulate the outer border, i.e., the surgical margin, may be expected to have a stronger EPR effect.

Because of the retention of ICG in tumor tissue, NIR imaging can locate residual nodules after surgery (Fig. 2A). Although there is substantial background fluorescence in the surrounding tissue, ICG uptake in tumors is more than 15-fold higher (Figs. 2B and 5). Attempts to diminish the dose of ICG decreased the background fluorescence, but also reduced the fold difference between the tumor and normal tissues (data not shown). Increasing the dose of ICG did not significantly improve imaging quality, thus our group chose to use 7.5 mg/kg. There was no concern of toxicity with ICG in this dose range as the LD₅₀ for this agent is in the order of 80 to 100 mg/kg. Synchronous labeling of murine cancer cells with GFP as shown in other models for imaging guided surgery (8) was also successful in our study to validate sensitivity for residual tumor tissue in our model (Fig. 2C).

NIR imaging systems such as the Li-Cor Pearl Impulse are useful for animal studies, however, our previously described handheld device is more practical for human application (17). In this trial, we found a handheld device to be significantly more sensitive than the Li-Cor Pearl Impulse. This is partially because of the ability to hold the device in close proximity to the tissue that is being examined. These studies raised several concerns that may limit this technology's full potential to locate tumor nodules. First, we acknowledge a small animal model of surgery is less than ideal to assess positive margins, particularly in the flank. However, we have created a reproducible model that can be conducted with consistent residual disease that is not palpable or visually obvious. Our preliminary studies with a spontaneous canine tumor model are promising. Second, the ICG is excreted via the biliary system, thus any tumors close to the liver cannot be assessed because of the high level of background. Third, we do not feel at this point we have sufficient data to conclude that 7.5 mg/kg is the best dose for visualizing residual disease. We have had similar success with smaller doses as well as using multiple doses, which will be addressed in future studies. In addition, it is not clear if the size of the original tumor could correlate with the fluorescence of tumor margins. Unfortunately, this is one significant limitation of this model and will likely need to be addressed in larger animal models or humans. Fourth, we emphasize that ICG is limited in clinical scope because of its nonspecific nature. It diffuses into any regions of vascular permeability, hence, both inflammatory and neo-plastic areas are likely to be highlighted. The surgeon will be required to use alternative clues to judge whether the area should be removed. Finally, depth of penetration in imaging ICG will be a limiting factor for more complex resections where the lesion of concern is behind other tissues. However, this study was conducted using ICG because it is currently the only FDA-approved contrast agent. Alternative particles do exist which permit deeper tissue penetration, but they would require higher excitation energy sources. For purposes of margins, however, this approach is feasible and efficacious as shown in this work.

This approach toward imaging intraoperatively had other advantages that should be acknowledged. In addition to assessing tumor margins, it is accurate for "back table" assessment after resection (Fig. 3C). Although frozen section will remain the gold standard, this approach has a superb advantage in rapidly examining residual tissue before delaying the case for pathologic analysis. In conclusion, this work adds a novel approach to rapidly assessing the surgical wound for residual disease with low toxicity, high sensitivity, and may add to the surgeon's armamentarium in judging completeness of resection.

Disclosure of Potential Conflicts of Interest
S. Nie is a consultant of SpectroPath, Inc., a startup company in Atlanta, GA, to develop advanced instrumentation and nanoparticle contrast agents for image-guided surgery. No potential conflicts of interest were disclosed by the other authors.

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Conception and design: B. Madajewski, D. Holt, M.D. Wang, S. Singhal Development of methodology: B. Madajewski, B. Judy, A. Mouchli, M.D. Wang, S. Nie, S. Singhal Acquisition of data (provided animals, acquired and managed patients, provided facilities, etc.): B. Madajewski, B. Judy, V. Kapoor, S. Singhal Analysis and interpretation of data (e.g., statistical analysis, bioinformatics, computational analysis): B. Madajewski, S. Singhal Writing, review, and/or revision of the manuscript: B. Madajewski, A. Mouchli, D. Holt, S. Nie, S. Singhal Administrative, technical, or material support (i.e., reporting or organizing data, constructing databases): B. Madajewski, B. Judy, S. Nie, S. Singhal Study supervision: S. Singhal

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Intraoperative Near-Infrared Imaging of Surgical Wounds after Tumor Resections Can Detect Residual Disease

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