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An Integrated Widefield Imaging and Spectroscopy System for Contrast-Enhanced, Image-guided Resection of Tumors

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Abstract

Tumor recurrence following surgery is a common and unresolved medical problem of great importance since surgery is the most widely used treatment for solid-mass tumors worldwide. A contributing factor to tumor recurrence is the presence of residual tumor remaining at or near the surgical site following surgery.

Goal—The primary objective of this study was to develop and evaluate an image-guided surgery system based on a near infrared, handheld excitation source and spectrograph in combination with a widefield video imaging system.

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A. M. Mohs, M. C. Mancini, and S. Nie are inventors of instrumentation related to this manuscript, which have been licensed to Spectropath, Inc. and may be subject to royalties and shares associated with the technology. S. Nie is an unpaid consultant to Spectropath, Inc.

Methods—This system was designed to detect the fluorescence of near infrared contrast agents and, in particular, indocyanine green. The imaging system was evaluated for its optical performance and ability to detect the presence of indocyanine green in tumors in an ectopic murine tumor model as well as in spontaneous tumors arising in canines.

Results—In both settings, an intravenous indocyanine green infusion provided tumor contrast. In both the murine models and surgical specimens from canines, indocyanine green preferentially accumulated in tumor tissue compared to surrounding normal tissue. The resulting contrast was sufficient to distinguish neoplasia from normal tissue; in the canine surgical specimens, the contrast was sufficient to permit identification of neoplasia on the marginal surface of the specimen.

Conclusion—These results demonstrate a unique concept in image-guided surgery by combining local excitation and spectroscopy with widefield imaging.

Significance—The ability to readily detect ICG in canines with spontaneous tumors in a clinical setting exemplifies the potential for further clinical translation; the promising results of detecting neoplasia on the marginal specimen surface underscores the clinical utility.

Index Terms

Image-guided surgery; ICG; indocyanine green; optical imaging; optical spectroscopy; surgical oncology; tumors

I. Introduction

Complete surgical removal of a tumor is one of the most critical predictors of a favorable outcome for an oncology patient [1]. To accomplish complete tumor resection, a surgeon must accurately determine the tumor boundary before and during surgery [2]. Presently, surgeons primarily rely on preoperative imaging and unaided intraoperative techniques (e.g., tactile and visual distinction of tumor from non-tumor tissue) to determine tumor boundary. One limitation of using only preoperative imaging is that tissue morphology can dramatically change intraoperatively. For instance, during brain tumor surgery, tumor shape and location can substantially change after removal of a portion of the cranium due to the mass effect of the tumor [3]. Preoperative imaging offers limited guidance in such circumstances. Reliance on tactile and visual qualities of the tumor is also of limited utility because small foci of tumor tissue frequently remain undetected, allowing residual tumor to remain in the operative bed (so-called “dirty margins”) [4]. Local tumor recurrence is one of the major causes of recurrent disease and tumor-related death [5–8]. Alternatively, the tactile and visual properties of normal tissue can simulate those of tumor tissue, leading to unnecessary resection of normal tissue. Improved identification of tumor margins in the surgical cavity, such that these lesions could be removed at the time of the surgical procedure, would likely improve patient outcome by reducing the rate of recurrence.

An improvement in intraoperative distinction of tumor from non-tumor tissue is an active area of interest by many investigators. Both intraoperative imaging through sonography [9] or magnetic resonance imaging (MRI) [10] have been used for intraoperative guidance. Sonography has many advantages: relatively low cost, compact nature of the equipment,

portability of the imaging device, video-rate imaging, lack of need for exogenous contrast agents, and ability for imaging probe placement within the surgical cavity. Furthermore, deformation of tumor shape has little impact because the sonography probe can be easily redirected. A significant disadvantage that ultimately limits intraoperative sonography use is that the contrast between tumor and normal tissue is low. Intraoperative MRI, on the other hand, uses a scanner placed adjacent to the surgical table and the patient is intermittently moved into the MR scanner. This technique allows identification of changes in tumor morphology and position. Unfortunately, use of such scanners is cumbersome because the patient is connected to a wide variety of monitoring and life-support equipment, which must be moved along with the patient. Furthermore, because the surgical procedure must be interrupted to allow for imaging, operative time and the time during which the patient is under general anesthesia are prolonged, which incurs increased risk to the patient and additional financial cost [11]. Intraoperative MR equipment also has substantial capital costs, e.g., cost of purchase and upkeep of equipment and cost of specialized room design and construction materials to maintain a safe imaging environment.

Intraoperative guidance to better define sites of tumor without prolonging surgery is an unmet clinical need. The ideal intraoperative guidance system would: (1) find tumor boundaries with high sensitivity, (2) have minimal impact on operative time and surgical technique, (3) present findings in an intuitive manner, and (4) avoid the use of ionizing radiation or a specialized imaging environment (such as MR imaging). Additional considerations in the design of an intraoperative imaging system would optimally include a compact, low-cost, portable system that was durable. Neither intraoperative sonography nor intraoperative MRI completely meets these design requirements.

Devices based on optical methods can meet the full range of the aforementioned design requirements. The near infrared (NIR) light range, between 700–1000 nm, is of greatest use for this application: NIR light undergoes less scatter than visible light in biological tissue and there is minimal biological background autofluorescence [12]. Silicon CCD and CMOS sensors have appropriate sensitivity for NIR detection. Likewise, fiber optic components and optical elements that are optimized for the NIR range are readily available at low costs. In combination with a NIR fluorescent imaging agent that can localize in tumor, these intraoperative imaging system design requirements are met. Here, we report the design and initial pre-clinical testing of an intraoperative imaging system that uses a handheld probe for simultaneous excitation and spectroscopic detection of NIR fluorescent contrast agents in combination with a widefield video-rate camera system for intuitive visualization. Optical imaging systems based on detecting contrast-enhanced fluorescence emission have been developed, including point imaging and widefield imaging systems [13–18]; we present an improvement over the current state of the art by incorporating aspects of both systems for optimized intraoperative tumor detection.

II. Methods

A. Integrated Optical Imaging System

1) Handheld Spectrograph Probe—The near-infrared (NIR) spectrograph with a remote handheld probe that we used in this study has been previously described and

characterized [13]. In brief, a coaxial fiber-coupled Raman spectrometer remote head (RamanProbe, InPhotonics, Norwood, MA) was coupled to a bench-top spectrograph (Advantage 785, DeltaNu, Laramie, WY). The excitation fiber of the remote probe was directly coupled to the laser diode module of the spectrograph (785 nm, 100 mW). The collection fiber of the remote probe (200 μm in diameter) was placed at the entrance to the spectrograph. The spectrograph was supplied with software to control excitation and data collection.

2) Widefield Video Camera—The widefield camera design targets were as follows: 45 cm working distance from target to lens, 5 cm object height, a 1/2 inch diagonal camera sensor at the imaging plane, and a collimated light path for filtering. Overall system design placed the highest priority on NIR light sensitivity to detect NIR fluorescent contrast agents at medically relevant concentrations and at a video-rate (30 frames/sec) acquisition speed.

The cameras (Guppy F-038B series, Allied Vision Technologies, Exton, PA) we employed used 1/2 inch uncooled and interlaced silicon CCD sensors with 47% quantum efficiency in the NIR range (as reported by the manufacturer). Cameras were connected to the computer by a Firewire 400 interface, which provided camera power and control. Sensor resolution was 768×492 , with a pixel size of $8.4 \mu\text{m} \times 9.8 \mu\text{m}$. The sensors used for each channel are the same; however, the anatomic camera includes a Bayer mosaic filter to provide color imaging and the NIR and laser channel cameras delete an NIR light filter (resulting in greater NIR light sensitivity).

The overall optical system design was a relay lens system that served as a link between a common objective lens and the camera sensor. We designed a collimated light path between the objective lens and camera sensors for placement of plate dichroic filters. Each channel of the system (e.g., anatomic, laser, probe) imaged a different, increasingly longer wavelength light range. Thus, the net track length per channel was not identical. An ideal optical design was first constructed under the assistance of an optical layout package (OSLO Version 6, Sinclair Optics, Pittsford, NY). Readily available commercial parts approximating the components of the ideal design were then substituted, evaluated and optimized using the optical layout package. The design process proceeded iteratively until the final design was reached, which we report in order of element encountered from object to image plane.

A C-mount lens, designed for broadband imaging between 400–1000 nm with a 35 mm focal length and $f/1.9$ maximum aperture (Xenoplan $f/1.9/35\text{mm}$, Schneider Optics, Hauppauge, NY), served as the common objective lens. This commercial objective was modeled as a perfect lens during system design because a lens prescription was unavailable. The field lens, designed to extend the objective lens focal length, consisted of a convex lens with focal length of 60 mm, a positive meniscus lens with focal length 100 mm, and a biconcave lens with focal length of -50 mm; this field lens group was then collimated with an achromatic doublet with focal length of 45 mm. An adjustable iris was located between the biconcave lens and achromatic doublet to improve image quality by rejecting rays that are far outside the paraxial region. The field lens group and collimating lens were coated with a magnesium fluoride (MgF_2) antireflection layer optimized for 700–1000 nm. The common objective, field lenses, and collimating lens were mounted in a single lens tube and

secured with screw-in retaining rings, in that order. Two dichroic mirrors were mounted sequentially in the collimated light path using kinetic mirror mounts. The first mirror was a short-pass dichroic mirror with a center wavelength cut-on of 800 nm, designed to direct light from detected contrast agent to the probe channel camera and allow laser excitation light or visible light to pass. A second short-pass dichroic mirror followed the first, with a center wavelength of 700 nm; the second dichroic mirror divided the remaining collimated light towards the laser channel camera or anatomic (i.e., visible light) channel camera. Beyond the dichroic filter system, the collimated light passed through a channel-specific series of filters before being focused onto the camera sensor with an achromatic doublet having a focal length of 45 mm.

For the probe channel, the collimated light was then filtered through a rugate notch filter centered at 785 nm to reject Rayleigh scattered laser light, using a rejection of optical density of at least 6. A bandpass filter with center wavelength of 820 nm and a 25 nm bandwidth was employed to reject all light except that from the contrast agent and provide supplemental blocking of Rayleigh-scattered laser light. For the laser channel, the reflected light was filtered through an absorptive neutral density filter having an optical density of 2 that was coated with an MgF_2 antireflection layer optimized for 700–1000 nm. The final component, the anatomical reference channel that allowed visible light, required no additional filtering beyond that provided by the dichroic mirror system.

All components, i.e., camera, focusing lens, and supplemental filters, were mounted in lens tubes, each of which was secured to a breadboard using lens tube slip rings and standard optomechanical hardware. The slip ring mount permitted simpler focusing of each individual channel. The system was made compact by incorporating a silver mirror between the objective lens tube and dichroic mirrors. The source of all lenses and optomechanical components was Thorlabs (Newark, NJ), except for the dichroic mirror mounts, which were obtained from New Focus (Newport, Santa Clara, CA) and the dichroic mirrors, which were obtained from CVI-Melles Griot (Rochester, NY). Two consumer-grade “white” 40 W LED lamps were used to illuminate the surgical field.

3) Widefield Imaging Software—The widefield imaging software was written for the Microsoft.NET platform using the C# programming language and Visual Studio Express development environment (2008 edition, Microsoft, Redmond, WA). An application programming interface (API) provided by the camera vendor (ActiveFirePackage, Allied Vision Technology) was used for camera control and data acquisition. This program uses an event-driven architecture by which each camera acquires frames in a detached execution thread and applies image filters after each frame is acquired. The contrast agent channel filter thresholds the image at a user-specified level to create a binary mask of contrast agent signal. The laser tracking channel filter thresholds the image at a user-specified level to create a binary mask of the laser position. The anatomical image channel filter combines the results of the contrast agent and laser tracking channel filters to produce a composite view presented to the user. This composite view displays the location of contrast agent signal as a cyan color (which was selected to be orthogonal to the colors of the surgical cavity) and displays the approximate position and size of the laser spot as a red circle outline. The software is able to record still frame images or live video (with MPEG-4 compression) for

documentation, as well as metadata about the experiment (*e.g.*, mouse tumor model used, contrast agent dose and type) in a plain text file. Parameters and specifications for the imaging system are listed Table 1.

B. In Vivo Murine Tumor Model Imaging

All *in vivo* murine studies were performed under a protocol approved by the Emory University Institutional Animal Care and Use Committee. Mice were age-matched athymic nude females (Harlan Laboratories, Indianapolis, IN). Tumor models were created with the 4T1 murine mammary carcinoma cell line (ATCC, Manassas, VA); cells were maintained as per ATCC recommendations. Prior to injection into mouse subjects ($N = 6$), the 4T1 tumor cells were washed (2x) with phosphate-buffered saline (PBS) before collecting and diluting to a final concentration of 2×10^7 cells/mL. Cells were injected into the flank, using approximately 2×10^6 cells per animal and allowed to grow subcutaneously for 28 days. At a time point 18–24 h prior to the imaging procedure, the NIR fluorophore indocyanine green (ICG, Sigma-Aldrich, St. Louis, MO) was infused within 10 s into the tail vein of mice bearing 4T1 tumors at a concentration of 357 $\mu\text{g/kg}$ (approx. 10 nmol per mouse). Immediately prior to imaging, animals were euthanized by CO_2 asphyxiation followed by cervical dislocation. The animals were then scanned with the spectroscopic pen at regular intervals, with the spectroscopic pen held a constant distance, 1 cm away, from the tissue by clamping the probe to a fixed stand and adjusting the position of the probe until a 1 cm distance was achieved as measured by calipers. NIR fluorescence spectra were collected and integrated using the trapezoid method. Simultaneously, video from the contrast agent, laser tracking, and anatomical channels were recorded using the widefield system. The threshold for the widefield imaging system was selected from mice that had been administered ICG. Mice were euthanized at 24 h and normal muscle, *e.g.* a contralateral flank or limb muscle, was placed under the image-guided surgery system. The NIR channel of the widefield was adjusted to a point which no NIR signal could be observed in normal muscle with laser excitation and then the threshold was set at 4 times that unit. The threshold setting obtained from the murine study was used in the canine study described below. NIR signal in normal muscle has a relatively small coefficient of variance (26.8%, see Suppl. Fig. 1). Surgical resection was then performed using standard techniques to remove the tumor mass using solely the unaided eye and palpation. Excised tissues, including tumor, tissue adjacent to tumor, and other areas of interest, were fixed in fresh 3.7% formaldehyde in PBS and submitted to the Winship Cancer Institute Pathology Core Laboratory for paraffin-embedding and processing using H&E stain or immunohistochemistry.

C. Canine Spontaneous Tumor Imaging

Companion dogs with a clinically diagnosed tumor were enrolled in a Clinical Research Committee-approved protocol at the University of Georgia College of Veterinary Medicine. A veterinary oncologist (CFS) recruited the dogs and obtained informed consent from dog owners, to whom a small financial incentive was offered for participating in the trial. To participate in the trial, canine subjects were required to have cytologically- or histologically-confirmed malignant solid-mass tumors amenable to surgical resection. Canines with severe underlying disease or a known allergy to contrast agents were excluded from enrollment in the study. Canine patients received standard of care treatment with addition of an

intravenous ICG infusion 18–24 h before surgery followed by monitoring for post-infusion anaphylactic reactions.

D. Ex Vivo ICG Accumulation in Companion Canine tumors and Pathologic Correlation

After *en bloc* removal of the tumor, the participating surgeon (KKC) obtained measurements of the surgical cavity using the spectroscopic pen placed in a sterile sleeve. Following surgery, tissue specimens resected from the patient, including biopsies of the tumor bed, were probed with the spectroscopic system in the presence of the attending pathologist (EWH), keeping a 1 cm distance between probe and tissue as described in the murine studies. Locations of spectral readings were recorded on a photograph of the tissue sample for later correlation to the histologic preparations, or when available, recorded by the integrated imaging system. The pathologist was blinded to the results of the spectral readings.

III. Results

A. Conceptual Overview of Intraoperative Imaging Platform

The intraoperative imaging platform is presented conceptually in Fig. 1 and consists of two instruments capable of detecting NIR fluorescent contrast agents. The first instrument is a point excitation source that is interactively positioned by the surgeon to direct excitation light in any area of interest. This source may be a broadband light source, such as provided by a xenon or LED lamp, or a monochromatic light source, such as a laser. In the case of an LED or laser, the source can be a self-contained handheld unit (like a laser pointer) or remotely fiber-coupled to the handheld unit. When fiber-coupled, the device can incorporate a spectrograph in order to provide detailed spectrographic information of the point being interrogated, as described in detail below. The second instrument is a widefield imaging system that sits the surgical field. The widefield imaging instrument has multiple area imaging sensors joined through a common lens. Each camera images a distinct spectral window provided by a system of optical elements. In the system that we describe here, we include one camera for color imaging of the surgical cavity (i.e., the anatomic view), one camera to track the point excitation source, and one camera for depicting the fluorescence of the contrast agent.

B. Current Implementation of Imaging Prototype

The current implementation of the imaging platform technology is pictured in Fig. 2. The point spectroscopy and excitation source consists of a commercially available Raman spectrograph fiber-coupled to a commercially available coaxial remote probe, described in further detail elsewhere [13]. The ICG detection lower limit of the spectroscopic unit is approximately 50 pM [13]. The widefield imaging system uses CCD cameras with high NIR sensitivity to depict the anatomical view, excitation source position, and to detect NIR light emitted by the contrast agent. The widefield imaging system is currently mounted on a flexible, articulating mount. The cameras are connected by FireWire to a computer running software developed in-house for image processing and display to the system operator. Similarly, the spectrometer is connected to the same computer for spectrograph acquisition using commercial or in-house software for processing and display. The display software

overlays all areas of the contrast agent above a set threshold onto the anatomic image, the centroid of the point excitation and displays this location as an empty circle on the anatomic image, and depicts the spectroscopic readings of the point of interest recorded from the handheld probe. The camera system channels are aligned using an ISO 12233 test target.

C. Workflow of Imaging System for Surgical Guidance in Mouse Models

The designed workflow of the integrated imaging system in cancer surgery in a typical subject is shown in Fig. 3. For initial proof-of-concept studies, we used the 4T1 murine breast cancer cell line injected into the flank of athymic nude mice to produce an ectopic tumor. As prepared, the mice develop a visible and fibrous-encapsulated tumor within days. In a typical experiment such as this example, 357 $\mu\text{g}/\text{kg}$ of ICG was infused into the mouse via the tail vein for imaging after 18 h. At the beginning of surgery, the tumor area is scanned with the handheld probe to determine the tumor boundaries. Tumors had 6.8-fold stronger ICG signal compared to muscle ($p = 0.0118$), see Supplementary Figure 1. The tumor is then resected with standard surgical technique.

Following resection, the surgical bed is rescanned with the handheld probe to assess for residual disease. If residual disease was found, the lesions detected by the imaging system are visually marked by pin placement, and then resected. The process is repeated until residual disease is no longer detected. The surgical specimens are then probed intraoperatively with the system to identify areas of particular interest for postoperative pathology. The guidance provided by the imaging system is unobtrusive: available when the surgeon needs to locate tumor boundaries and residual disease, and out of the surgical field while removing tissue or performing other surgical duties.

In one example, the 4T1 tumor grew in an ectopic fashion with the mouse skin. Thus, when the skin flap was retracted from the surgical cavity, the primary tumor remained engrafted with the skin, as shown in Fig. 4. Consistent with the subject shown in Fig. 3, ICG preferentially accumulated into the primary tumor as shown Fig. 4B. Following the surgical workflow, reexamination of the surgical area indicated an unexpected, a “hot spot” of high ICG accumulation in an area distant from the fibrous tumor capsule. On visual and tactile inspection, this tissue appeared to be connective tissue. The specimen was removed and submitted for pathology: histological examination confirmed that this “hot spot” was in fact a lymph node with metastatic tumor cells as determined by CK-18 staining. Supplementary Fig. 3 shows that the widefield imaging system can detect high contrast-enhancing regions and low contrast-enhancing regions as the handheld pen is moved across these regions.

D. Image-Guidance in Spontaneous Canine Tumors

Murine tumor models have a distinct fibrous capsule and frequently an exceedingly leaky vasculature; consequently, they do not fully replicate the human tumor microenvironment morphologically or physiologically. To demonstrate the utility of ICG for contrast-enhanced tumor imaging with the current intraoperative imaging prototype in a more realistic biologic and clinical setting, we tested the system by imaging companion dogs with spontaneously occurring solid tumors. Canine patients were systemically administered 220 $\mu\text{g}/\text{kg}$ ICG 24h before surgery. Tissues removed from the patients were analyzed by the intraoperative

imaging system in a room adjacent to the surgical suite. An example of results obtained from a canine procedure is shown in Fig. 5. Tumor to muscle contrast enhancement was 7.3, which was larger than expected and likely due to the size of this individual tumor. Additionally, ICG was detected in an area that appeared to be normal muscle but was determined by pathology to be inflammation-associated edema. A distinct boundary of ICG accumulation could be observed between tumor tissue (as determined by visual and tactile inspection) and normal tissue bordering the specimen.

IV. Discussion

Surgery remains a widely used and effective treatment of solid tumors. Despite numerous advancements in diagnostic imaging, few technological innovations have been directed towards *intraoperative* guidance of tumor resection. Diagnostic imaging techniques like CT, MRI, and PET/SPECT provide *preoperative* information as to the location and distribution of solid-tumor masses, but once surgery begins, changes in the surgical field (such as shift in organ location and change in tumor morphology following partial removal of tissue) limit the utility of preoperative imaging in guiding a surgical procedure. In particular, preoperative imaging cannot inform on the status of a surgical margin (i.e., has a tumor mass been completely removed). Attempts to develop MRI as an intraoperative modality are hampered by numerous limitations in use: restrictions on ferrous tools, moving the patient to permit imaging, bulky MR imaging scanners, and the prohibitive expense of intraoperative MR imaging equipment. Pathology techniques such as touch-prep and frozen section histology are greatly limited in throughput and can solely examine surgical specimens after removal, rather than the surgical bed [19, 20]. The frozen section process takes on the order of 20 minutes, thereby causing delay in completion of surgery and prolonged intra-operative times. Furthermore, the delay to obtain final histological diagnosis is much longer, i.e., on the order of 48 hours, well after the completion of the surgical procedure. The ability to provide a definitive intraoperative diagnosis would not solely decrease the time for decision-making. It would, most importantly, provide information that would allow a surgeon to incorporate results while still in the operative suite and thus, make a decision whether to resect additional tissue.

We have developed an optical intraoperative imaging system that relies on systemically injected contrast agents to provide intraoperative guidance during surgery. Imaging with optical contrast material has no ionizing radiation concerns (as with CT/PET/SPECT), does not place restrictions on tools that can be used in the operating theater (as with MRI), and the instrumentation can be made compact, portable, and durable at a lower cost than CT, MRI, or PET/SPECT. Recently, optical intraoperative imaging systems have been developed which use area excitation with widefield imaging [15], [21], [22]. In contrast, we use point excitation with widefield imaging. This scheme allows for spectroscopy to be obtained in real-time and for an intuitive widefield imaging display to offer surgical guidance. Spectroscopic capabilities are useful with fluorescent contrast agents to distinguish fluorescence emission of the contrast agent from endogenous fluorophores or external stray light. In the future, intraoperative spectroscopy can be used with surface-enhanced Raman scattering (SERS) based contrast agents to provide both higher sensitivity and multiplexed imaging [13], [23].

Due to a lack of suitable commercial instrumentation, we developed a prototype to perform our study, with a goal of clinical translation of both the instrumentation and the protocols for use. The overall system consists of three parts: an exogenous contrast agent, a point excitation source, and a widefield imaging system. The intraoperative imaging platform requires a contrast agent to be infused into the patient prior to imaging. This contrast agent can be an organic fluorophore (e.g., indocyanine green), a fluorescent nanoparticle (e.g., a semiconductor quantum dot), or a Raman-active agent (e.g., a surface-enhanced Raman scattering (SERS) gold nanoparticle). For initial studies, we selected indocyanine green (ICG) as the contrast agent. ICG is well-suited for these preclinical studies for several reasons: it is FDA-approved for blood pool monitoring, it has a good safety profile with well-defined counter indications [24], and it has absorbance and emission in the “near-infrared window” where endogenous chromophores have low absorption and there is low autofluorescence. Recent publications have featured ICG used for sentinel lymph node (SLN) mapping with intraoperative imaging instrumentation [25], [26], however, there are few reports of the use of ICG as a contrast agent for determining tumor boundaries intraoperatively [14], [27–29]. Since ICG is known to correlate well with the blood pool, and tumors are known to have a “leaky” vasculature [30–32], it is reasonable to expect that ICG will accumulate within tumors and could be used to delineate the tumor boundary.

Experiments in murine tumor models show that ICG deposits in higher amounts in the tumor relative to surrounding tissues, which can be delineated by the image-guided surgery system as Figs. 3 and 4. In the ectopic mouse tumor model, ICG was shown to provide sufficient tumor contrast to be identifiable on our system, as well as in one case, allowing for identification of a lymph node with metastatic disease that would have been unnoticed without this intraoperative imaging system. While the small animal model demonstrates that the concept of ICG as a tumor contrast agent has merit, it introduces many difficulties in conducting a full correlative study of ICG accumulation in tumors to determine if ICG can truly be used as a tumor *boundary* contrast agent. The difficulties are largely those of scale, but there are also the additional questions posed in using a tumor model to test an imaging agent that relies on enhanced permeability and retention (EPR) effect for “targeting” since mouse tumor models are well-known to have leaky vasculature that is not representative of naturally-occurring human tumors. To overcome these difficulties, we started a clinical trial in companion canines with spontaneous tumors. Canine tumors are known to be similar to human tumors in architecture, and canines get the same types of tumors as humans (although in different occurrence rates) [33]. Another advantage of using canine patients is that ICG has a history of use in canines and is both known to be safe and to behave similarly as it does in humans [34], [35]. Surprisingly, we found that intravenous infusion of ICG in canines accumulates in spontaneous tumors as it does in the mouse tumor models. An important implication is that this suggests that ICG is likely to provide contrast for human tumors as well.

The widefield imaging technique and local spectroscopy technique each have individual advantages. Local excitation and spectroscopic detection yields wavelength-resolved information that allows one to distinguish endogenous versus injected fluorophores. The handheld fiber-coupled laser and spectrometer used here can acquire NIR spectra in 0.1 s

[13] in the areas of interest in the surgical field. Spectroscopic information via a widefield imaging system would require tunable filters (which are inefficient and costly), which would not be conducive to video-rate imaging. Furthermore, the directed laser allows the operator to unobtrusively collect light over a large solid angle. Because the surgeon can direct the laser to the point of interest, numerous computer video processing schemes can be used to assist in imaging, e.g., delineating the tumor boundary on the widefield imaging screen by tracking the handheld probe position, or zooming the widefield imaging system display on the handheld probe position. Another advantage of this scheme is that any fluorescent dye outside the inspected area is not at risk of photobleaching. Finally, the local excitation source is a practical way to use a relatively high power (but permissible under ANSI criteria) laser for illumination in the operating room to achieve high excitation fluence. Using a laser excitation source is desirable both to achieve high fluence rates (both high relative intensity and a source that can be focused into a small area) and to provide a monochromatic light source that can be rejected in the widefield imaging system fluorescence channel with notch filters. Using area illumination for the same purpose would require high power LED or laser diodes that would color the surgical field and introduce electrical and optical safety issues. Alternative light sources for fluorescence excitation, such as Halogen or xenon, are broadband and require extensive optical filtering to prevent bleed-through into the emission channels on widefield imaging. The relatively high fluence inherent in the point excitation source allows for the widefield imaging system to have a straightforward design if the optical layout and component selection are biased in favor of near-infrared sensitivity. In the instrumentation presented in this article, we were able to use uncooled CCD sensors and catalog optical parts, which would not be possible with an area illumination scheme with an inherently lower fluence rate. To that end widefield imaging is essential for intuitive surgical guidance. It provides real-time spatial guidance for NIR contrast-enhancing areas. We are currently developing a second-generation system with a smaller widefield imaging head unit, less intrusive mounting, and improved monitor position.

V. Conclusion

The utility of ICG for imaging tumors with low-cost instrumentation amenable to intraoperative use has been demonstrated. Our technology addresses the issue of minimization of the likelihood of postoperative residual tumor, which is a major cause of tumor recurrence. The resultant better delineation of tumor boundaries during surgery, such that the tumor can be removed *in toto*, is expected to greatly increase the cure rate of surgical resection. Future studies will be needed to more rigorously establish the correlation between ICG fluorescence and the histologically-defined tumor boundary in order to support human clinical trials. Additionally, studies will need to be conducted that focus on the development of higher-performance fluorescent dyes and SERS nanoparticles, which can be targeted to specific tumor markers.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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Fig. 1.

Operational scheme of widefield imaging with directed point excitation and spectroscopy. The widefield imaging head (center-right) is positioned over the surgical area. Directed point excitation is provided in this case by the handheld probe (S). The widefield imaging system uses 3 cameras to image the anatomical view (A), the laser position within the anatomical view (L), and any near-infrared (NIR) light emitted within the anatomical view (N). The area is imaged by a common objective lens (O) and the collected light is folded by mirrors (M) and filtered by dichroic mirrors (D). Complex optics within the widefield imaging head (e.g. collimation lenses, bandpass filters, etc.) are not depicted. The three video channels recorded by the cameras (top-right) and spectral information collected by the handheld probe (bottom-center) are processed by a computer system to synthesize a composite display (bottom-left) to show anatomical landmarks with the laser position and any detected NIR signal.

Fig. 2.

Photographs of current device implementation. Shown in (A) is the widefield imaging head. The objective and collimation optics (orange box) are housed in a lens tube mounted 90° from the rest of the components: a 45° protected-silver mirror folds the light to the rest of the system. The dichroics (magenta box) are used for separating the collected light into the NIR (blue box), laser (red box) and anatomic (green box) channels, in that order. Each channel has additional filters and focusing optics mounted in lens tubes and then directly coupled to the camera. Shown in (B) is the handheld probe held in typical use pattern along side several common surgical implements. Panel (C) shows the spatial relationship between the handheld and widefield units. In the operating room a member of the surgical team held the pen.

Fig. 3.

General workflow for resection of tumors by interactive intraoperative guidance. The images on the left and right of each panel show the same mouse with the handheld probe in two positions at each stage of tumor removal. Initially, the suspect tumor area is scanned to find the borders of the tumor (a). The tumor is then removed by standard surgical technique. Following resection, the surgical bed is rescanned to find residual ICG signal (b). The resection is revised until no ICG signal is detected in the surgical bed (c). Finally, the resected surgical specimen is scanned to confirm ICG accumulation and areas of pathologic interest for histologic analysis (d). Areas of ICG accumulation are false-colored cyan, and the area the point excitation source is being directed is shown by the dashed red circle. Suppl. Fig. 2 shows the spectral signal in representative area of these images.

Fig. 4.

Systemically injected ICG preferentially deposits in tumors but not normal tissue. A nude athymic mouse bearing a subcutaneous 4T1 xenograft mammary tumor was injected by tail vein with 357 $\mu\text{g/kg}$ ICG 24 h before dissection and imaging. (A) When the handheld probe is directed away from the tumor, no ICG emission is detected by the widefield imaging system or spectrometer and (a) histology is consistent with skeletal muscle. (B) When the handheld probe is directed onto the tumor, the widefield imaging system is able to detect ICG emission (cyan false-color) and the spectrometer is able to resolve ICG emission, while (b) H&E histology of the enhancing region is characteristic of tumor. (C) The widefield imaging system also detects ICG emission when the handheld probe is directed onto an abnormal lymph node, which was confirmed by spectroscopy. (c) Histology of the lymph node with CK-18 immunohistochemistry is positive for metastatic tumor cells (yellow arrows). Scale bar = 200 μm . Suppl. Fig. 3 shows the wide-field channel as the handheld pen system is moved on and off tumor and lymph node.

Fig. 5.

Post-operative analysis of a surgical specimen from a canine with spontaneous fibrosarcoma. A 13-year old mixed-breed canine was infused with 220 $\mu\text{g/kg}$ ICG 24h before surgery to amputate the left hind leg that had an 8.5 cm in diameter mass. (A) The leg was dissected post-operatively for analysis of normal and neoplastic tissue as determined by visual and tactile inspection. ICG was found to accumulate and deposit in the tumor (C, cyan false-colored region) but not fatty connective tissue (E) or normal muscle (B). (F) Areas rich in ICG deposits (tumor) show a unique spectral signature that is distinct from the broad, featureless spectrum of normal tissue (muscle, fat). (D) A slight increase in ICG signal was observed in inflamed tissue associated with the tumor, consistent with reports of ICG presence in inflammation-associated edema. Suppl. Fig. 4 shows the widefield channel as the handheld pen system is moved through the field.

Table I**Intraoperative Imaging System Specifications**

	Parameter	Specification
Widefield Detection	Number of imaging channels	3
	Imaging wavelengths	400–700, 700–800, 800–900 nm
	Camera resolution	768–492
	Camera maximum frame rate	60 fields/s (interlaced)
	Camera sensor size	½ in
	Camera cell sensor size	8.4 $\mu\text{m} \times 9.8 \mu\text{m}$
	Camera NIR QE	700 nm: 80%, 800 nm: 47%, 900 nm: 23%
	System resolution limit	4 lp/mm (horizontal), 2.8 lp/mm (vertical)
	Intended working distance	60 cm
	Field of view (at working distance)	11 cm $\times$ 7 cm
	Pixel spatial resolution (at working distance)	145 μm
	System aperture	$f/4$
	ICG detection limit	150–500 pM
Spectrographic System	Excitation source wavelength	785 nm
	Excitation source max irradiance	1.3 kW/cm ²
	Excitation source min spot size	100 μm
	Spectrograph resolution	8 cm ⁻¹ (0.6 nm)
	Spectrograph range	200–2000 cm ⁻¹
	ICG detection limit	50 pM
	Dynamic range	40–50 fold