Real-time, Near-Infrared Fluorescence Imaging with an Optimized Dye/Light Source/Camera Combination for Surgical Guidance of Prostate Cancer

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Translational Relevance

Robot-assisted laparoscopic prostatectomy is completely dependent upon visualization to assure complete removal of malignant tissue. Here we present a technique that enhances the capacity to provide a negative surgical margin after prostatectomy using a near-infrared fluorescent compound we recently described in conjunction with a light source specifically tuned to its detection in the near infrared region of the spectrum. A negative margin avoids the morbidity experienced by patients who often undergo irradiation if a positive margin is identified.
Introduction

Radical prostatectomy is the gold standard in the surgical treatment of prostate cancer (1). Despite advances in technique, surgery for prostate cancer remains fraught with positive surgical margins (PSM) due to the inability to detect early extra capsular extension intra-operatively (2). Efforts to decrease PSM by the use of numerous technical maneuvers during the procedure have been disappointing, suggesting that new techniques are needed to prevent residual malignant tissue at the time of surgery, when it can still be removed (3). The proximity of the neurovascular bundles and rectum to the prostate precludes routine wide excision during radical prostatectomy, and efforts to spare the neurovascular bundles (and preserve penile erectile function) results in PSM in 10-38% of men at high volume centers, with particularly high rates in men with the most dangerous and aggressive disease (2, 4). Men with PSM are more likely to suffer biochemical recurrence (2) and require adjuvant radiation therapy (5), with increased morbidity and expense as a consequence (6).

Every PSM causes the patient to undergo salvage radiation therapy, which adds an additional $25,000 - $35,000 to their care. In some reports, robotic prostatectomy patients have as high as a 3.7-fold higher risk of a PSM, than open surgery (7). Hu et. al. reported an estimated 27.8% of men undergoing robotic prostatectomy underwent salvage radiation, compared to 9.1% with open surgery. Given that currently more than 233,000 men are diagnosed with prostate cancer each year and more than 54,000 men undergo robotic assisted prostatectomy as their primary treatment, as many as 15,000 men undergo adjuvant radiation therapy primarily because of an issue with PSM. The estimated cost to our government is >$375M for radiation therapy alone and more than $1B in other costs from the impact of that
treatment. Therefore novel technologies that limit PSM during prostatectomy may decrease the morbidity and expense associated with surgery for prostate cancer.

Advances in the sensitivity of imaging techniques and the development of new fluorophores with high quantum yields have raised interest in real time fluorescence guided imaging for use in surgery. Recent work has focused on fluorophores in the NIR range (wavelengths between 700 nm and 900 nm), due to the low absorption and autofluorescence of biologically relevant molecules in this range, particularly hemoglobin and water (8). Thus, in a surgical field in which blood and bodily fluids are present, signal from the NIR fluorophore should remain readily visible. These fluorophores permit a high signal-to-noise ratio due to the low level of autofluorescence, thus depicting target cells as bright stars in a dark background. Use of this technology relies on the development of fluorescent probes that specifically target cancer cells as well as imaging modalities that provide accurate real-time imaging.

Prostate-specific membrane antigen (PSMA) is a type II transmembrane glycoprotein that catalyzes the hydrolysis of N-acetylaspartylglutamate (NAAG) to glutamate and N-acetylaspartate (NAA) through an extracellular domain. PSMA is markedly over-expressed in virtually all malignant prostate tissue and expression increases with tumor aggressiveness (9). In addition, PSMA has been identified in the neovasculature of nearly all solid tumors, including colorectal cancer, bladder cancer, glioblastoma multiforme, breast cancer, pancreatic cancer, and testicular cancer (10, 11). Accordingly, PSMA is emerging as an important target in cancer imaging and therapy (12-16). We have previously described the development and use of YC-27, a novel low-molecular-weight fluorescent agent (absorbance maximum 774 nm, emission maximum 792 nm) that targets PSMA and enables near-infrared (NIR) imaging of cells expressing PSMA in murine models of prostate cancer in preclinical testing (17). Here we report
the use of a proprietary laparoscopic imaging system (LumiNIR™) designed to detect NIR fluorescent agents \textit{in vivo} that consists of a novel NIR laser light source utilizing a wavelength optimized for YC-27, and a modified low light Charged Couple Device (CCD) camera, which is operated similarly to a conventional white light laparoscopic platform. When combined with YC-27, the LumiNIR™ system allowed detection of very small burdens of PSMA-positive cells \textit{in vitro} with minimal signal from PSMA-negative cells. In a murine model, PSMA-positive xenografts were readily detectable after intravenous (IV) administration of YC-27, even allowing visualization of PSMA-positive tumors through the skin in real time. In a porcine laparoscopic model that closely approximated human laparoscopic extirpative surgery, a PSMA-positive xenograft was clearly identified and removed using the LumiNIR™ system. The use of the LumiNIR™ along with a targeted dye (YC-27) allows for refinement of current surgical techniques to decrease PSM.

Materials and Methods

Cell Culture.

PSMA-expressing LNCaP cells were obtained from ATCC (Manassas, VA. Cat# CRL1740). Both the PSMA-expressing PC3-PIP and PSMA-negative PC3-FLU cell lines were obtained from Dr. Warren Heston. LMD and LMD-PSMA cells were generated by lentiviral transduction of cells derived from lung metastasis of mice bearing orthotopic MDA-MB231 tumors that were provided by Dr. Sridhar Nimmagadda (Supplmentary Methods). All cells were cultured in RPMI 1640 medium (Corning Cellgro, Manassas, VA. Cat# 10-040) with 10% FBS (Sigma-Aldrich, St. Louis, MO. Cat# F4135-500ML), 50 ug/ml of Gentamicin (Quality
Biological, Gaithersburg, MD. Cat# 120-099-661) and 5 ug/ml of Ciprofloxacin (US Biological, Salem, MA. Cat# C5074). Cells were passaged at confluency of 90% at a dilution of 1:10. Cell cultures were maintained in 5% CO2, at 37°C in a humidified incubator.

**Authentication of cell lines.**

All cell lines used in the study were tested for the lack of mycoplasma using the MycoAlert™ Mycoplasma Detection Kit (Lonza, Basel, Switzerland) every six months. The LNCaP cells were authenticated by STR analysis. The PC3-PIP, PC3-FLU, LMD-PSMA and LMD cells were characterized by their PSMA expression status and cell morphology.

**Immunohistochemistry.**

The resected tumor was fixed using formalin, embedded in paraffin and sectioned. Histologic analysis was performed using hematoxylin and eosin stained slides. Immunohistochemistry for PSMA expression was completed using a Ventana automated immunostainer and an i-View immunolabeling kit (Ventana Medical Systems, Tucson, AZ). For antigen retrieval, the specimen was incubated with Standard CC1 (EDTA buffer) for 60 minutes, followed by incubation with the primary antibody (PSMA clone 3E6, 1:100 dilution (30 minutes) at 37° C; DAKO Cytomation, Carpentaria, CA).

**Animal models.**

All animal experiments were approved by the Animal Care and Use Committee at our institution and performed in accordance with relevant guidelines and regulations.
Resection of Murine Orthotopic Prostate Cancer.

Under inhalational anesthesia with a mixture of isoflurane (Baxter Healthcare Corporation, Deerfield, IL) and oxygen, a lower abdominal incision was made in an Athymic Nu/Nu mouse. To establish an orthotopic prostate cancer model, 50 μL of a 1:1 suspension of 300,000 PC3-PIP cells and Matrigel™ (BD Biosciences, San Jose, CA. Cat# 354248), was injected into the dorsal lobe of the prostate, and the animal was closed. 16 days later, 79.4 μg/Kg of YC-27 was administered via tail vein injection. 24 hours after injection an exploratory surgery was performed using the Fluobeam™ (Fluoptics, Grenoble, France), a near infra red (NIR) imaging system. The abdomen and pelvis were carefully inspected for fluorescent signal. NIR imaging was performed at 5 to 15 frames per seconds (fps) (the Fluobeam™ automatically adjusted the frame rate based on the signal intensity). The orthotropic tumor was identified by its fluorescent signal and resected.

Epididymis imaging.

Athymic Nu/Nu mice were injected with 79.4 μg/Kg of YC-27 and 24 hrs later imaged with the Pearl®. Animals were euthanized and organs harvested, washed once with 1x PBS and imaged on the Odyssey®. Images were captured at 700 and 800nm at a resolution of 21 mm using the same parameter settings. The 800nm channel was displayed using a pseudocolor output and overlaid on the grayscaled 700nm channel in Adobe Photoshop CS4, Version 11.0 (San Jose, CA).

RT-PCR for PSMA.
Six athymic Nu/Nu mice were sacrificed, and organs were harvested. RNA was isolated from kidneys, liver, and epididymis using TRIzol reagent (Invitrogen, Carlsbad, CA. Cat# 15596-026). 1mg of total RNA was reverse transcribed using QuantiTect Reverse Transcription Kit (Qiagen, Valencia, CA. Cat# 205311) to generate cDNA. Intron/exon spanning mouse PSMA specific primers were used to detect PSMA message in the different organs. 250ng of cDNA was used in PCR analysis using GoTaq Green Master Mix (Promega Corporation, Madison, WI. Cat# M712) according to manufacturer’s protocol.

Proprietary Laparoscopic NIR Imaging System with the LumiNIR™ light source.

A proprietary 5.5 W 870 nm class 4 laser was designed and fabricated (Patent pending) and named LumiNIR™. The complete system is depicted in Supplementary Fig. S1. The light source was coupled with a Stryker 502-457-010 10mm 0º laparoscope (Stryker, Kalamazoo, MI) using a Karl Storz 495NCS light cable (Karl Storz, Tuttlingen, Germany). The laparoscope was adapted to the Prosilica GX1920 camera (Allied Vision Technologies, Stadtroda, Germany) using a c-mount Stryker 24mm coupler (Stryker, Kalamazoo, MI). The Prosilica GX1920 camera utilizes a Sony EXview HAD ICX674 sensor which has a quantum efficiency of ~31% at 800nm. A long pass filter, HQ800lp (Chroma Technology Corporation, Bellows Falls, VT), which allows light of 800nm or higher to pass through, was used in between the coupler & the sensor of the camera. The system is designed to allow the user to manually set the exposure time / frame rate, gain, and resolution during video and still image capture.

NIR-guided Laparoscopic Resection of Subcutaneous Tumor in a Murine Model.
A PSMA positive xenograft was established by injecting a 2:1 suspension of 3x10^6 LMD-PSMA cells and Matrigel™, into the right flank of Athymic Nu/Nu mice. When tumors reached a volume of ~0.5 cc, 19.1 μg/Kg YC-27 was administered via tail vein injection. NIR guided laparoscopic resection of the xenograft was carried out in a laparoscopic training case (Global trade, Hong Kong, Hong Kong), using the LumiNIR™ 8 - 10 hrs post IV injection. Images were taken prior and post resection at 5 fps.

**Visualization of porcine kidney after YC-27 administration.**

A 30 kg male yorkshire pig was anesthetized with 1.32 ml of TKX (Tiletamine HCl 50mg/mL; Zolazepam HCl 50mg/mL; Mannitol 57.7mg/mL; Ketamine 50 mg/ml; xylazine 50 mg/ml) given IM. To suppress pain and prevent infection, 0.6 mg of buprenorphine and 660 mg of cefazolin was administered IM. After intubation, a Veress needle was inserted into the peritoneal cavity, and CO₂ was used to insufflate the peritoneum to 15 cm H₂O. Three 10 mm ports were placed under laparoscopic vision. YC-27 was administered via intravenous injection at a dose of 3.33 μg/kg YC-27 and the kidney was observed over 30 minutes. Laparoscopic images were recorded with the LumiNIR™.

**Detection of a prestained xenograft placed inside a porcine abdomen.**

After establishing a 1000mm³ LMD-PSMA xenograft in an Athymic Nu/Nu mouse, 19.1 μg/Kg YC-27 was injected IV 24 hrs prior to the pig surgery. A 30 kg male yorkshire pig was anesthetized with 1.32 ml of TKX (Tiletamine HCl 50mg/mL; Zolazepam HCl 50mg/mL; Mannitol 57.7mg/mL; Ketamine 50 mg/ml; xylazine 50 mg/ml) given IM. To suppress pain and prevent infection, 0.6 mg of buprenorphine and 660 mg of cefazolin was administered IM. After
intubation, a Veress needle was inserted into the peritoneal cavity, and CO₂ was used to insufflate the peritoneum to 15 cm H₂O. Three 10 mm ports were placed under laparoscopic vision. The murine YC-27 pre-stained tumor was surgically removed and placed behind the porcine peritoneum adjacent to the kidney. The pre-stained xenograft was visualized and removed with the guidance of the LumiNIR™

**Optimization of Timing and Dose.**

A 2:1 suspension of 3x10⁶ LMD in Matrigel™ was injected into the upper flanks of 20 Athymic Nu/Nu mice while an equivalent LMD-PSMA suspension was injected into the contralateral flank. When the tumors reached a volume of ~0.5 cc, YC-27 was administered via tail vein injection in varying doses (39.7, 19.1 and 9.5 μg/Kg). Mice were sequentially imaged at 1, 2, 3, 4, 6, 8, 10, and 24 hrs post IV injection using the LumiNIR™ at 5 fps. The images were captured at a constant gain setting using the “GigE Viewer” (Allied Vision Technologies GmbH, Stadtroda, Germany) to obtain probe kinetics. The GigE Viewer interface allows the user to control the exposure time/frame rate and gain settings of the camera to the user’s preference. Regions of interest were drawn around tumor sites, kidneys, muscle, and background. Arbitrary pixel count was used to evaluate dosing and timing for signal-to-noise ratio using ImageJ (18).

**Comparing the LumiNIR™ System to the Pearl®.**

A 2:1 suspension of 3x10⁶ LMD and Matrigel™ (BD Biosciences, Billerica, MA), was injected into the upper flanks of Athymic Nu/Nu mice, while an equivalent suspension of LMD-PSMA was injected into the contralateral flank. When the tumors were ~200mm³ 79.4 μg/Kg YC-27 was administered IV. 24 hours post injection the animals were scanned on the Pearl® Impulse...
imager (Li-COR, Lincoln, NE) at 170 micron resolution according to manufacturer’s protocol, which requires about 30 seconds for image capture. Immediately afterwards, the animals were imaged using the LumiNIR™ at 10 fps and images were captured using the “GigE Viewer” (Allied Vision Technologies, Stadtroda, Germany). Images captured on the Pearl® and the LumiNIR™ were analyzed using the Pearl®’s software and ImageJ (18) respectively.

**Comparing the LumiNIR™ System to da Vinci.**

Dilutions of YC-27 and ICG (Sigma, St. Louis, MO. Cat# I2633) with 1x10^4 LMD-PSMA cells were plated into a flat bottom black 96 well plate in duplicates and layered with 100 μl of 1% agarose. Two plates were made and one shipped to Intuitive on ice while the other was kept at 4°C. Both plates were imaged about the same time (48 hrs post plating). One was imaged with the da Vinci® Si™ System with the Firefly™ attachment, that utilizes a proprietary image capture system which has a 1080p HD output and the exposure time / frame rate cannot be manually adjusted. The other was imaged with the LumiNIR™ at 5fps. Arbitrary pixel count was analyzed with image J (18). Wells containing cells but no dye were used to determine the noise of the systems.

**Results**

**Detecting a PSMA-positive orthotopic tumor in a mouse.**

We had previously described the development and use of YC-27, a low-molecular-weight fluorescent agent that targets PSMA and enables near-infrared (NIR) imaging of cells expressing PSMA in murine models of prostate cancer (17). Utilizing the Fluobeam™ (Fluoptics, Grenoble,
France), a commercially available preclinical NIR imaging system, we set up a more realistic surgical situation. To determine if YC27 is able to identify and aid in resection of orthotopic murine prostate tumors, $3 \times 10^5$ PC3-PIP cells were implanted in the dorsal lobe of the prostate of athymic Nu/Nu mice. Sixteen days post implantation 79.4 µg/Kg YC-27 was administered via intravenous (IV) injection. Twenty-four hours after IV administration of YC-27, exploratory open surgery with the aid of the Floubeam™ was performed and a 3 mm orthotopic prostate cancer xenograft was detected and surgically resected under NIR guidance (Figs. 1A & 1B). Hematoxylin and eosin staining of the xenograft confirmed the presence of malignant cells (Fig. 1C), and immunohistochemistry with an anti-PSMA antibody proved that they were derived from the PSMA-positive PC3-PIP xenograft (Fig. 1D). Interestingly, signal in the epididymis was observed, indicating YC-27 uptake, which was unexpected (Figs. 2A & B). Expression was confirmed by RT-PCR on RNA collected from the epididymis, kidney and liver of athymic Nu/Nu mice. The kidney was used as a positive control and, as expected, expression of murine PSMA mRNA was detected (19), while the liver provided a negative control (Supplementary Fig. S2). The Fluobeam™ is not compatible with current laparoscopic surgical equipment, which led us to developing our own imaging system.

**Development of the LumiNIR™ system.**

NIR imaging is based on exciting a fluorophore and detecting the light emitted after it is excited. In order to do this we engineered and fabricated an NIR detection system, LumiNIR™, which can easily be integrated into both laparoscopic and robotic equipment (Supplementary Fig. S1). The LumiNIR™ is comprised of a novel class 4 laser based off a 780 nm light source with a total power output of 5.5 watt, and a modified Prosilica GX1920 high resolution CCD camera.
NIR fluorescence images are captured using the ⅓" Sony ExViewHAD II ICX674 sensor in the Prosilica GX1920 with a reported 31% quantum efficiency at 800 nm modified with a 800 nm longpass filter in front of the sensor. The light source is engineered to provide more than 30 different angles of light oriented and focused to precisely enter a standard laparoscopic high density fiber optic light cable. When coupled to a properly coated laparoscope, the light is diffused to more than 45 degrees as it exits the laparoscope, resulting in a wide field of illumination without creating potential areas of heat injury to the host tissue.

Use of LumiNIR™ in animal models.

To test the LumiNIR™ system in a surgical setting we utilized small and large animal surgical models. Breast cancer cells engineered to express PSMA (LMD-PSMA) were subcutaneously xenografted into the right axilal of athymic Nu/Nu mice. Exogenous expression of PSMA did not appear to change the in vitro growth and migration characteristics when compared to the parental LMD cells (Supplementary Figure S3-6). When tumors reached a volume of 500mm³, mice were administered 19.1 μg/kg (HED of 100 μg for a 70 Kg male) YC-27 via tail vein injection and after a 10 hour uptake time, mice were placed in a laparoscopic training box where the xenografts were resected with the guidance of the LumiNIR™ system (Supplementary Movie S1). Supplementary Figure S7 shows images of the tumor bed before and after resection. Minimal signal in the tumor bed after resection indicated a possibility of residual tumor.

We then moved on to testing the system in a porcine model, an appropriate model for laparoscopic surgery to assess the feasibility of using YC-27 with the LumiNIR™. Within five minutes of administrating 3.33 μg/kg of YC-27, NIR signal was easily visible in the porcine kidney with the LumiNIR™ (Figs. 2A & 2B). This finding was expected, as YC-27 is known to
have renal clearance (17), and PSMA is expressed in the murine and human kidneys (19, 20). Expression in porcine kidney has not been extensively studied but there is one report that it has expression in porcine kidney (21). Additionally, the signal intensity from the kidney was directly proportional to the amount of excitation by the light source (Supplementary Movie S2). To assess whether tissue absorption of NIR signal would limit utility of NIR imaging in vivo, a LMD-PSMA tumor xenograft was explanted from its murine host after YC-27 administration and implanted behind the porcine peritoneum during laparoscopic exploration (Figs. 2C & 2D). To further demonstrate the utility of LumiNIR™, the pre-stained xenograft was easily visualized and removed through the guidance of the LumiNIR™ (Supplementary Movie S3).

**Performance of LumiNIR™ compared to a dedicated small animal imager.**

Prior characterization of YC-27 was done on the LI-COR Biosciences Pearl®, a dedicated small animal imager. To test the LumiNIR™ system and determine the optimal dose and timing for surgery, athymic Nu/Nu mice bearing contralateral PSMA-positive and negative tumors were injected intravenously with 39.7 µg/kg, 19.1 µg/kg, and 9.5 µg/kg of YC-27 and imaged at regular intervals using the LumiNIR™ (images for all concentrations of YC-27, and at all time points were captured under identical settings at 5 fps). The three different doses are based on the Human Equivalent Dose (HED) calculation (22) and would be approximately 2 x, 1 x and 0.5 x of what is considered a microdose in human studies (100 µg) (23). An initial whole-body, fluorescent blush was observed in all animals immediately after injection with clearance from non-target tissues, proceeding according to the anticipated bio-distribution kinetics (17) (Supplementary Fig. S8). Signal in the PSMA-positive tumor was dose-dependent and non-specific signal was cleared by 6 – 10 h post-injection, depending on the dose administered.
(Supplementary Fig. S9). The highest signal-to-noise ratio of 11.37 was observed 6 hours after administration of 9.5 μg/kg of YC-27, while at the dose of 19.1 μg/kg the signal-to-noise ratio was 10.94 at 8 hours post administration, and at the highest dose of 39.7 μg/kg the signal-to-noise ratio was 10.4 at 10 hours post administration.

To compare the LumiNIR™ to the LI-COR Biosciences Pearl®, athymic Nu/Nu mice bearing contralateral PSMA-positive and negative tumors of about 200mm³ were administered 39.7 μg/kg YC-27 via IV injection. The animals were imaged with the, LI-COR Biosciences Pearl® 24 hours post injection, and immediately afterwards using the LumiNIR™ system at 10 fps. Regions of interest (ROI) were drawn around the PSMA-positive and -negative tumors from images generated from the LumiNIR™ and LI-COR Biosciences Pearl® (Figures 3A-C), and signal-to-noise ratios were calculated. Despite differences in exposure time between the Pearl® imager and LumiNIR™ (approximately 30 seconds per image vs. 0.1 seconds, respectively), the signal-to-noise ratio was nearly the same with both systems (Fig. 3D), supporting that the real-time laparoscopic LumiNIR™ system possesses the sensitivity necessary for application to a clinical setting.

Performance of LumiNIR™ compared to da Vinci® Firefly™.
Currently, the only commercially available surgically guided NIR imaging system is the Intuitive Surgical NIR clinical device, the Firefly™ attachment to the da Vinci® robot. This is a system that is equipped with filters optimized for indocyanine green but can also detect YC-27. To compare the LumiNIR™ and da Vinci® Firefly™, identical plates containing dilutions of indocyanine green (ICG, peak emission wavelength 830 nm) and YC-27 (peak emission wavelength 792 nm) were imaged on both systems. To quantify the signal, regions of interest
(ROI) were drawn around each dilution series. In this setting, the LumiNIR™ could detect as little as 15.63 pmole of ICG, which is similar to the Firefly™ (Supplementary Fig. S10). When imaging YC-27, the LumiNIR™ was more sensitive than the da Vinci® Firefly™. When using a signal-to-noise ratio of 1.2 as a cut-off value for visual detection, the LumiNIR™ could detect 2.48 pg of YC-27 compared to 39.69 pg of the compound required for detection by the da Vinci® system (Fig. 4).

**LumiNIR™ can reduce positive surgical margin.**

The LumiNIR™ was developed with the goal of being able to reduce positive surgical margins. To determine if image-guided surgery using YC-27 and the LumiNIR™ as a means to reduce positive margins, we resected PSMA-positive xenografts after YC-27 administration with the guidance of LumiNIR™ system (n=8) or by conventional surgery (n=10). For animals resected under the guidance of the LumiNIR™, tissue was resected until there was no residual signal (Fig. 5). Upon white light evaluation there was no detectable residual tumor in any surgery. The animals were then followed for over a month, and only the animals resected by conventional surgery had recurrence at the primary site (Fig. 6 & Supplementary Table S1). The recurrence rate of 40% in the surgeries done under white light is in the range of what is seen with aggressive tumors. Treuting et. al. have seen 33% - 53.5% recurrence rates at the primary site after surgical resection of MDA-MB-435 xenografts in nude mice (24). We have previously seen 25% - 40% recurrence at the primary site after simple surgical resection with RenCa, CT-26, TrampC2 and B-16 xenograft models (unpublished data). These results demonstrate NIR image guided surgery utilizing the LumiNIR™ can be used to decrease positive surgical margins resulting in decreased morbidity and mortality. A reduction in PSM would also reduce the costs associated with
adjuvant therapies, and morbidities associated with them, which is the standard of care in patients with PSM.

Discussion

The primary objective in oncologic surgery is complete removal of malignant tissue at the time of resection. However, the ability of the human eye or even laparoscopically magnified imaging to discriminate malignant tissue from surrounding benign tissue often limits the completeness of resection, particularly for microscopic tumor burdens. The advent of tumor-specific, efficient NIR fluorophores and increasingly sensitive CCD sensors, promises to allow detection of these microscopic extensions of tumor at the time of surgery, when they may be readily excised (25). This paradigm-shifting technology may enable more complete excisions and reduce the need for expensive and morbid adjuvant therapies.

Intraoperative NIR fluorescent surgical guidance has recently become feasible for a variety of reasons. First, fluorescent emission may be captured quickly (~30 ms), allowing real-time detection with frame rates of up to 30 frames per second, consistent with laparoscopic and robotic surgical technology. Second, NIR fluorescence utilizes CCD cameras at high spatial resolution, allowing high definition images. Third, NIR fluorescence technology is readily compatible with existing laparoscopic and robotic surgical systems, and Intuitive Surgical has recently integrated the Firefly™ imaging technology (Novadaq Technologies, Mississauga, ON, Canada) into the da Vinci® system. The data presented in this study suggest the potential use of the Firefly™ attachment in augmenting tumor visualization during radical prostatectomy after administration of YC-27. Alternatively, the LumiNIR™ is optimized for detection of YC-27 and
provides even greater sensitivity to detect microscopic extension of tumors in vitro and may enable detection of smaller tumor burdens during oncologic surgery.

Sensitivity analysis found that YC-27 can distinguish as few as 3,125 PSMA positive cells over PSMA-negative cells in vitro (supplementary Fig S11 & S12). Considering that 1 mm³ of tumor is estimated to contain ~100,000 tumor cells (26), this sensitivity may allow detection of very small tumor burdens, as would be expected at the site of a positive margin or in micrometastatic disease. In murine models, a signal-to-noise ratio of approximately 11 was seen after intravenous injection of YC-27, and when combining this high signal-to-noise ratio with the excellent spatial resolution allowed by a CCD camera, tumor borders had sharp contrast with surrounding tissue in real-time imaging. A proof of concept study was performed in a relevant laparoscopic porcine model by visualizing and removing a prestained xenograft from behind the peritoneum of a male pig. The LumiNIR™ was also able to facilitate the surgical removal of PSMA-positive prostate cancer xenografts in a murine model and decrease the rate of positive surgical margins.

A similar urea-based agent has been used for Positron Emission Tomography/Computed Tomography (PET/CT) imaging in humans and detected more putative metastatic lesions than the gold standard combination of CT and bone scintigraphy with minimal toxicity (13), suggesting that the small doses of YC-27 likely necessary in humans will be effective with limited side effects. Studies performed with dual modality Single-photon emission computed tomography (SPECT)/optical (NIR) compounds demonstrate that with a single tracer dose minimal lesions could be detected (12). Using such compounds would allow a single administration to allow for SPECT imaging prior to surgery and fluorescent imaging during research.
surgery—with the potential benefit of preoperative surgical planning for potential sites of extracapsular extension and micrometastatic disease to local lymph nodes.

Previous studies have evaluated the use of near-infrared fluorescence imaging to guide detection and treatment of cancer, including ovarian cancer (27, 28), colorectal cancer (29), breast cancer (30), gastric cancer (31), head and neck cancer (32), and liver cancer (33). Van Dam et al evaluated intraoperative fluorescence imaging in ovarian cancer using a folate-FITC agent, which fluoresces in the visible spectrum and targets FR-\(\alpha\) (27). The authors detected all FR-\(\alpha\)-positive tumors intra-operatively, with a signal-to-noise ratio of 3.1:1. With the combination of LumiNIR™ and YC-27 we can obtain a signal-to-noise ratio of up to 11.3:1. According to Eckelman et al, the ideal signal-to-noise ratio for an imaging agent is 10:1 (34).

To date, most reports using the da Vinci®/SPY™ system have utilized non-specific NIR dyes (35), but recently, Laydner et al reported a proof-of-concept study using a PSMA-targeting small molecule and the da Vinci®/Firefly™ Surgical System in murine surgery (15). Weak signal was detected in 2 of 3 mice with large PSMA-positive xenografts. Though no signal-to-noise ratios were reported, background signal was noted to be strong, particularly from the kidney, liver, and bladder, which may limit utility during prostatectomy. Furthermore, a large quantity of the imaging agent was necessary to obtain these results (10 nmol, approximately 60 x a microdose) compared to 0.5 x microdose (0.12 nmole) of NIR fluorescent agent in the current study. Unfortunately, in the Laydner et al study the fluorescence signal detected was not reliable for surgical applications due to the weak signal despite large PSMA-positive xenografts. No signal was detectable from the base of the tumor, which questions whether the agent and technology could reliably detect PSM. In the current study, NIR-guided laparoscopic surgery
was accurate and reliable using very small doses of YC-27. Also in this study we demonstrate large animal applications that closely approximate human laparoscopic surgery.

We report a practical NIR dye/light source/camera combination that could be easily implemented in contemporary prostate cancer surgery without disrupting the common clinical pathway, offering a novel strategy for the detection and treatment of prostate cancer. We also compare our system to the da Vinci® Firefly™ attachment, the most widely used robotic surgical system. Our combination would enable exquisite detection of PSMA expressing cells and has been evaluated in relevant animal models, including simulation of human laparoscopic extirpative surgery. This technology can be used to detect minimal disease not infrequently left in the surgical bed after RALP, thereby minimizing or even eliminating a PSM and the attendant morbidity and cost of adjuvant therapy. The ability to potentially eliminate PSM during RALP may represent the single biggest cost savings achievable. Moreover as PSMA is expressed in most tumor neo-vasculature, this technology can also be extended to other malignancies to aid in tumor resection, where residual tumor portends not only morbidity and cost, but also increased mortality.

Acknowledgments: We would like to thank Dr. Jonathan Sorger of Intuitive Surgical for imaging the plate with varying concentration of YC-27 & ICG with their Firefly™ attachment for the da Vinci® Si™ system. We would like to thank Dr. Stoianovici of the URobotics lab for his help in fabricating the housing for the LumiNIR™ light source. We would also like to thank Dr. Patrick C. Walsh for his advice with the project. Funding: We would also like to thank the NIH RO1 CA134675, PSMA-Based Cancer Imaging Agents, and the Patrick C. Walsh Prostate Cancer Research Fund for a grant that partially funded this project.
References and Notes:


Figure Legends.

Figure 1: **Real time detection & surgical resection of a PSMA positive orthotopic xenograft.** Orthotopic tumors were established in the dorsal lobe of the prostate of Athymic Nu/Nu mice by injecting PC3-PIP cells. 24 hrs post YC-27 administration the animals were imaged and an established 3mm orthotopic tumor was surgically resected with the guidance of the Floubeam (A & B). Arrow points to PC3-PIP orthotopic tumor. Yellow arrow heads point to epididymis. The resected xenograft was formalin fixed & sections for staining, (C) H&E, (D) IHC for PSMA. The surgically removed tumor is shown in white light (inset panel B). In this experiment we had an N=3 animals.

Figure 2: **Detection of YC-27 in a laparoscopic setting.** (A) White light image of the porcine kidney, which was located during an exploratory laparoscopy. The NIR scope can be seen in the image frame. (B) NIR image of the porcine kidney after YC-27 administration taken with the LumiNIR™. (C) A prestained murine PSMA-positive xenograft placed behind the peritoneum of the pig could easily be identified using the LumiNIR™. (D) The same tumor seen in C could not be distinguished from the normal tissue in white light. N=1 pig, the xenograft experiment was repeated three times, while the Kidney was only done once.

Figure 3. **Comparing the LumiNIR™ to the Pearl® imager.** Athymic Nu/Nu mice (N=5) with established PSMA positive (white arrow) and PSMA negative (yellow arrow) subcutaneous tumors were imaged using the Pearl® (A), and the LumiNIR™ - PSMA positive (B) and PSMA negative (C) 24 hours post IV administration of 79.4 μg/Kg YC-27. Comparing the PSMA
positive & negative xenografts similar signal-to-noise ratios was observed with both the systems (D).

**Figure 4. The Da Vinci® System can be used to detect YC-27.** Identical plates containing dilutions of YC-27 were used to compare the LumiNIR™ to Intuitive Surgical’s NIR clinical surgical system, the Firefly™ attachment of the da Vinci® system. The Firefly™ has been optimized for compounds like the ICG, while the LumiNIR™ has been optimized for compounds such as the YC-27. The LumiNIR™ can detect as little as 2.84 pg of the compound while with the Da Vinci® system we could detect 39.7 pg of the compound. This demonstrates the ability to use the Da Vinci® if enough probe is used. It might be possible to detect tumors using tracer doses in a patient with an optimized detection system.

**Figure 5. Comparison of Surgeries With and Without NIR Illumination.** Animals with established LMD-PSMA xenografts were given 1 nmole YC-27 about 20 hours before the surgery. The first (leftmost) four columns contain photographs of surgeries performed using with NIR guidance so that there was no residual tumor left. The second (rightmost) four columns contain photographs of surgeries performed without NIR guidance. Under white light, both sets looked the same, i.e., it was impossible to determine whether there was residual tumor under white light. All the animals were imaged with NIR device described herein post-surgery and with the Pearl® imaging system after the animals were sutured. The animals were also imaged seven days post-surgery with the Pearl® imaging system. 1 nmole YC-27 was administered intravenously the day before the imaging. We resected tumors on 8 animals with
the guidance of the LumiNIR™, while there were 10 other animals on which the surgical resection was carried out under white light. This experiment was carried out only once.

**Figure 6. Comparison of tumor free survival post surgery aided by LumiNIR™ or under white light.** Athymic Nu/Nu mice harboring PSMA-positive xenografts were administered 19.1 μg/kg (HED of 100 μg for a 70 Kg male) YC-27 via tail vein injection. In a subset of the animals (8) the tumors were resected with the guidance of LumiNIR™ system. In the rest of the animals (10) the tumors were resected under white light. The animals were examined for palpable tumors over time. All animals were imaged with the LumiNIR™ and the PEARL® immediate post surgery. The animals were also imaged 7, 14, 21 & 30 days post surgery, each time 19.1 μg/kg YC-27 was administered via tail vein injection 6 hrs prior to imaging. The results are summarized in supplementary table S1.
Figure 2:
Figure 3

Fold Signal over Background

<table>
<thead>
<tr>
<th>Method</th>
<th>LMD</th>
<th>LMD-PSMA</th>
<th>LMD</th>
<th>PEARL</th>
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<td>LumiNIR</td>
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<td>1</td>
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<tr>
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<td>PEARL</td>
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Figure 4

LumiNIR vs. Intuitive System

Pixel Count

Signal to Noise

YC-27 [pg]
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<tr>
<th>Surgery Performed using LumiNIR™</th>
<th>Surgery Performed under White light</th>
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</table>

**Figure 5**
Figure 6

Tumor Free Survival %

LumiNIR

White light

Time [Days]
Clinical Cancer Research

Real-time, Near-Infrared Fluorescence Imaging with an Optimized Dye/Light Source/Camera Combination for Surgical Guidance of Prostate Cancer

Brian P. Neuman, John B. Eifler, Mark Castanares, et al.

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