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Real-time, intraoperative detection of residual breast cancer in lumpectomy cavity walls using a novel cathepsin-activated fluorescent imaging system

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Introduction

Breast conserving surgery, also termed lumpectomy, provides survival equivalent to that of mastectomy for most women with breast cancer [1, 2]. Modern lumpectomy with clean margins, followed by radiation and systemic therapy provides excellent local control, lowering the risk of in-breast recurrence to approximately 2-3% for most histological subtypes [3]. This degree of local control is important, as it is now recognized that local recurrence can decrease survival, with 1 excess death for every 4 breast cancer local recurrences [4].

Unfortunately, achieving microscopically tumor-free margins needed to prevent local recurrence is challenging. Preoperative imaging does not accurately reflect microscopic tumor anatomy [5], and standard surgical techniques still result in positive lumpectomy margins in 20-40% of patients [6, 7, 8, 9, 10]. These positive margins require a second surgical procedure to excise additional breast tissue to obtain tumor free margins, which increases patient discomfort and anxiety, worsens cosmetic outcomes and adds to the cost of care.

All currently available options for detecting positive lumpectomy margins during the initial surgery have significant limitations [11]. Frozen section histopathology analysis of margins reduces positive margin rates in some series [12, 13] but prolongs surgery, is expensive, is

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All procedures performed in studies involving human participants were in accordance with the ethical standards of the institutional and/or national research committee and with the 1964 Helsinki declaration and its later amendments or comparable ethical standards.

Informed consent was obtained from all individual participants included in the study.

not widely available and allows for analysis of only a small fraction of the lumpectomy surface during the initial surgery. Imprint cytology (touch prep) analysis is highly sensitive with specificity of 65-100% [6], but requires cytopathology expertise not available at most institutions.

Approaches for more rapid and effective intraoperative lumpectomy margin assessment are needed. Experimental approaches, including micro-computed tomography scanning [14, 15], spectrally encoded confocal microscopy [16] and optical coherence tomography [17] have not yet produced clinically applicable results. In prospective trials, the MarginProbe® device, which uses radiofrequency spectroscopy for margin assessment, reduced the absolute rate of second surgeries by 6% [18, 19] with a 25% false negative rate [18], but these results have not led to widespread clinical use.

Most approaches for reducing positive margins focus on enhanced methods for imaging the surface of excised lumpectomy specimens. This approach is inherently flawed in that it does not determine the location of residual tumor in the lumpectomy cavity wall, which is where the residual tumor is located. Rather, it identifies tumor on the surface of a soft, pliable lumpectomy specimen, whose geometry no longer accurately corresponds to the geometry of the cavity from which it was excised [20].

The ideal approach for intraoperative margin assessment for cancer surgery would rapidly identify residual tumor directly in the walls of the surgical cavity, guide additional excision and verify that clear margins have been achieved. We now describe the use of a system with these properties for intraoperative detection of residual tumor during breast cancer lumpectomy surgery.

The Lumicell (LUM) Imaging System uses (1) a novel PEGylated protease-activated far-red fluorescent imaging agent, LUM015 [21], (2) a hand-held probe for intraoperative tissue imaging [22] and (3) software for image analysis. In a Phase 1 study in 15 human patients [21], this system distinguished malignant sarcoma and breast cancer tumor tissue from surrounding normal tissue in *ex vivo* surgical specimens. We now report the first human *in vivo*, intraoperative use of the LUM Imaging System.

Methods

The Lumicell (LUM) Imaging System (Lumicell, Wellesley, MA) includes LUM015, a novel PEGylated protease-activated far-red fluorescent imaging agent [21]; the LUM optical head, a hand-held probe and sterile cover used to excite LUM015 and collect real-time fluorescent recordings; and software for image analysis. The LUM Imaging System excites activated LUM015 with a 630nm LED source. Fluorescent photons are collected by a charge coupled device (PCO AG, Germany) after passing through an emission filter and imaging lens. Fluorescent signal values are reported in 10^{10} counts/s/cm^2. The LUM Imaging System probe has a 2.6 cm diameter circular field of view and is covered by a sterile plastic sleeve for intraoperative use.

We conducted an IRB-approved, prospective, non-randomized, open-label study at Massachusetts General Hospital, Boston, MA. Eligibility requirements included age ≥18

years, invasive breast cancer or ductal carcinoma in situ (DCIS) diagnosed by needle biopsy, and planned lumpectomy surgery. Exclusions included prior ipsilateral cancer surgery, neoadjuvant systemic therapy, other serious concurrent illnesses, a prolonged corrected QT interval, current pregnancy, allergy to LUM015 components, inability to provide informed consent or inability to complete study requirements. Subjects agreed to use contraception for 60 days after surgery. Complete blood count (CBC) and comprehensive metabolic panel (CMP) lab tests were performed preoperatively and at the first post-operative visit.

Sequential enrollment accrued 5 subjects not injected with LUM015, 5 injected with 0.5 mg/kg of LUM015, and 5 injected with 1.0 mg/kg of LUM015. LUM015 was injected as a 3-minute intravenous push 4±2 hours prior to surgery. Wire localization for non-palpable lesions, and Technetium-99 injection for sentinel lymph node biopsy was performed before or after LUM015 injection. Methylene blue used for sentinel node mapping can produce fluorescent signal similar to LUM015 and was not used prior to lumpectomy, but could be injected after lumpectomy and evaluation of the cavity with the Lumicell Imaging System at the surgeon's discretion.

Lumpectomy cavity walls were imaged with the LUM Imaging System probe *in vivo* and excised lumpectomy specimens and excised shaved margins specimens were imaged *ex vivo.* Breast autofluorescence was assessed in patients who did not receive LUM015. Tumor to normal tissue (T:N) fluorescent signal ratios were determined by transecting excised lumpectomy specimens *ex vivo* and using the probe to image the cut surface where tumor and normal tissue were present. LUM015 fluorescent signal and histopathology features were co-localized for regions of tumor and normal tissue as identified by study pathologists. T:N tissue signal was assessed in patients receiving LUM015 and used to refine tumor detection software algorithms. Safety data was collected for all patients.

Routine histopathological evaluation was performed following current diagnostic standards [23] Hematoxylin and eosin stained slides were reviewed by the study pathologists (EFB, TRS) to correlate microscopic images with the Lumicell fluorescent signals. Invasive carcinoma was categorized as invasive ductal or lobular type, or invasive carcinoma with mixed ductal and lobular features.

All study interventions were completed at the end of the surgical procedure. Patients were discharged from the hospital the day of surgery and assessed for adverse events at the first post-operative visit.

Given the small size of this study, a Wilcoxon rank-sum test was used to determine whether it is equally likely that a point chosen from the T:N values in the 0.5 mg/kg dose cohort is less than or greater than a point chosen at random from the 1.0 mg/kg dose cohort. The null hypothesis is rejected for a p-value < 0.05. This analysis was performed using the wilcox.test function in R software (R Foundation for Statistical Computing, Vienna, Austria). One-way analysis of variance tests (ANOVA) were performed using the *aov* function in R. ANOVA analyses were performed to test the null hypothesis that patient-level data sets could have been extracted at random from a single pooled data set.

Results

Ex vivo breast autofluorescence

Thirteen breast specimens were obtained from 9 breast surgery patients aged 34-65 years who did not receive LUM015, and imaged *ex vivo* using the LUM Imaging System. Far red shifted light was used to measure autofluorescence in the 700nm wavelength. Specimens examined included 8 mastectomies, 4 lumpectomies and 1 re-excision and were used to obtain 123 LUM images 34±7 minutes after excision. Malignant specimens imaged included invasive ductal cancer, invasive lobular cancer, DCIS, carcinoma in situ with ductal and lobular features and positive axillary nodes. Benign tissues imaged included benign breast parenchyma, fibrocystic change, fibroadenoma and healing biopsy site changes, as well as nonobligate precursor lesions such as atypical ductal hyperplasia, lobular neoplasia and flat epithelial hyperplasia. Background fluorescence was seen in areas of injection of methylene blue dye used for sentinel node identification. The average signal intensity on the surface of specimens from the 6 patients that did not receive methylene blue for sentinel node injection was 1.85×10^{10} counts/s/cm^2. No significant background autofluorescence was observed in any of the normal tissue in cancer containing specimens.

LUM015 dose escalation

In the prospective dose escalation trial, 15 breast cancer lumpectomy specimens had intraoperative lumpectomy cavity imaging and *ex vivo* specimen imaging with the LUM Imaging System. Five patients received no LUM015, 5 patients received 0.5 mg/kg and 5 received 1.0 mg/kg of LUM015 as a single bolus dose intravenously 4±2 hours prior to surgery. Median patient age was 63 years (range 48-78 years). Eleven patients had invasive carcinoma (ductal or lobular) with associated DCIS and 4 subjects had pure DCIS. Breast density on mammography was heterogeneously dense in 10 of 15 patients. Patient and tumor details are presented in Table 1.

Breast autofluorescence was assessed intraoperatively using the LUM Imaging System probe in the lumpectomy cavity and on excised specimens of the 5 study patients who did not receive LUM015. Image acquisition for each 2.6 cm diameter surface required approximately 1 second, with areas of fluorescent signal displayed on a computer screen for viewing by the surgeon.

In patients who did not receive LUM015 there was no significant *in vivo* normal tissue background autofluorescence signal detected in the 700nm wavelength relative to signal obtained in the 0.5 and 1.0 mg/kg dose cohorts. Mean signal from the in vivo lumpectomy cavity walls was: $1.31 \pm 1.16 \times 10^{10}$ counts/s/cm^2 in patients who did not receive LUM015; $10.96 \pm 5.93 \times 10^{10}$ counts/s/cm^2 in patients who received 0.5 mg/kg LUM015; and $10.11 \pm 5.55 \times 10^{10}$ counts/s/cm^2 in patients who received 1.0 mg/kg LUM015.

LUM Imaging System performance

The LUM015 imaging agent produced fluorescent signal that distinguished areas of tumor from normal tissue at both 0.5 and 1.0 mg/kg doses. Mean T:N signal ratios were 4.70 ± 1.23 (n=5) and 4.22 ± 0.96 (n=4) at 0.5 mg/kg and 1.0 mg/kg, respectively, with no

statistically significant difference between doses (p=0.54) (Table 2). There was some tumor fluorescence seen in patients who did not receive LUM015, with mean T:N ratio 2.05 (Fig1).

Elevated fluorescence was seen in invasive cancers with ductal, lobular and mixed ductal and lobular histology and for pure DCIS (Fig2). The calculated T:N signal ratio was lower for pure DCIS and for invasive tumors with extensive associated DCIS, likely related to the difficulty of assessing signal in the small diameter areas of DCIS (Table 2).

Tumor could be distinguished from surrounding normal tissue in both pre- and postmenopausal women and did not appear to be significantly impacted by breast density as measured on mammography. Table 1 shows T:N signal ratios and patient and tumor characteristics for all study patients.

Some benign tissues produced fluorescent signal with LUM015 (Fig3) sometimes with signal levels as high as seen in areas of that patient's tumor. Histological evidence of active fibrocystic change and areas of inflammation were seen microscopically in some of these areas, but only normal breast tissue was identified in others. Elevated normal tissue signal did not appear to correlate with menopausal status or breast density in this small series.

The event rate of positive margins in this small study was low, with only 4 pathology-confirmed positive cavity wall margins in the 0.5 mg/kg dose cohort and 1 in the 1.0 mg/kg cohort. As a result, analysis in this study focused on variation among patients. For each of the subsets of data investigated, p-values were generated to determine the likelihood that imaging data from each patient in that subset could have originated from the same population. For each *in vivo* and *ex vivo* data set presented in Figure 3, p-values below 0.05 were observed in testing the null hypothesis, suggesting that each patient in a given cohort could not have likely been pulled at random from an aggregate data set.

Safety

There were no adverse events attributed to participation in this study. One patient had transient hypertension on induction of anesthesia and again on awakening that study monitors deemed unlikely to be related to LUM015 or use of the imaging probe. All subjects were discharged from the hospital the day of surgery. There were no intraoperative or post-operative complications thought to be related to study participation, including no surgical site infections. All patients had blue discoloration of their urine for approximately 24 hours after surgery, related to excretion of the LUM015 dye. Blood tests performed at the routine postoperative visit showed no clinically significant abnormal measurements related to study participation.

Discussion

Obtaining microscopically tumor-free margins is the central goal of all cancer surgery. Current margin assessment approaches for breast cancer lumpectomy surgery look for tumor on the surface of excised lumpectomy specimens. Histopathology assessment is slow, with permanent pathology results taking several days. Frozen section testing of margins, which can add 20-30 minutes to a surgical procedure, is costly and not widely available. Standard

and current experimental approaches also suffer from challenges related to specimen deformation that makes orientation unreliable, trauma to specimen surfaces that results in false positive margins, and the inability to assess more than a fraction of a specimen surface [11, 20, 24], With current technology, 20-40% of breast cancer lumpectomy patients have positive surgical margins that require a second surgery to achieve clear margins [6,7, 8, 9, 10].

A cavity-based margin assessment strategy that allows direct identification of residual tumor in the patient's surgical cavity would overcome many of the limitations of current specimen-based approaches. In this study, we tested the Lumicell Imaging System, a cavity based margin assessment technology. The LUM015 dye is a protease activated agent that is administered intravenously prior to surgery and becomes fluorescent in areas containing tumor [21]. The fluorescent signal is detected by a sterile probe that is inserted in the surgical cavity, with areas of tumor signal displayed on a computer monitor for viewing by the surgeon.

The LUM Imaging System fulfilled the goals of (1) safety, (2) high sensitivity for tumor detection, (3) rapid assessment of the entire lumpectomy cavity, and (4) precise identification of sites of tumor in the lumpectomy cavity wall. No study patient had adverse effects attributed to LUM015 injections or use of the Lumicell probe during surgery.

The LUM Imaging System was sensitive for distinguishing benign and malignant breast tissue in transected tumor specimens and, most importantly, could identify islands of residual tumor against a background of normal tissue in lumpectomy cavity walls and excised tissue specimens. We found no significant background autofluorescence in benign human breast tissue, supporting the use of a fluorescence-based strategy for lumpectomy margin assessment.

LUM015 produced good tumor:normal signal ratios in both premenopausal and postmenopausal patients and was not affected by breast density as measured by mammography. Previously documented tumor autofluorescence [25, 26] did not impact performance of the LUM Imaging System in distinguishing tumor from benign tissue. Invasive ductal cancers, invasive lobular cancers and ductal carcinoma in situ (DCIS) could all be distinguished from surrounding normal tissue. We did observe lower tumor:normal signal in some DCIS specimens, likely related to the small cross sectional area of ducts containing DCIS. We are exploring the option of a DCIS-specific detection algorithm to be assessed in future studies.

Although the event rate of positive margins in this small study was low, an analysis of variance (ANOVA) performed on the *ex vivo* and *in vivo* data from the two dose cohorts suggests that patients would be best served by an algorithmic approach that takes into consideration each patient's normal tissue baseline signal. In a follow-up study, an algorithm will be developed to set a patient-specific threshold separating benign from tumor signal and highlight areas of residual tumor for excision by the surgeon.

Use of the LUM Imaging System was feasible in the operating room setting with minimal alteration of surgical workflow. The Lumicell probe and cover were similar to other devices

used during breast surgery, such as sentinel node gamma probes. Image acquisition for each 2.6 cm diameter area of lumpectomy cavity wall required only 1 second, allowing complete imaging of an entire lumpectomy cavity in a minute or less. By comparison, the MarginProbe® field of view is 0.7 cm, requiring 5-8 measurements per margin surface and requiring 5 minutes or more to assess the surface of an excised lumpectomy specimen [18, 19]. Other experimental devices also have the limitation of only 0.5-1.0 cm diameter fields of view [16, 17].

Most importantly, the LUM Imaging System directly identifies sites of residual tumor for excision, and can be used repeatedly until no residual tumor signal remains. This cavity-based approach avoids the specimen-based problem of correlating sites of tumor on an excised lumpectomy surface with the location of residual tumor in the cavity wall.

This pilot study is limited by the small number of breast cancer specimens studied. Additional evaluation of the efficacy of the LUM Imaging System for obtaining clear margins during breast cancer surgery is underway, and use of the system for other cancers is being explored.

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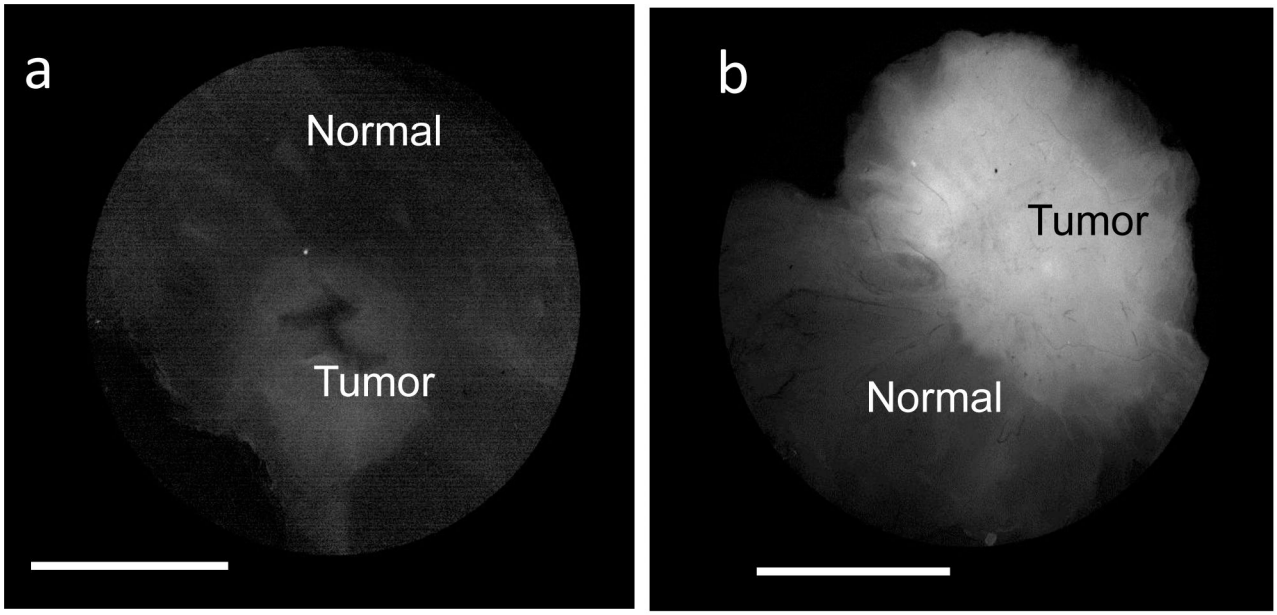

Figure 1:

LUM Imaging System images of transected tumors with and without LUM015 injection: (a) Transected lumpectomy specimen from Patient 5, IDC and DCIS, no LUM015 with tumor:normal signal ratio of 1.9 (b) Transected tumor specimen from Patient 8, IDC and DCIS, 0.5mg/kg LUM015 with tumor:normal signal ratio of 4.8. Images are plotted on linear brightness scales for which the minimum pixel value is black and the maximum pixel value is white. Scale bars = 1 cm.

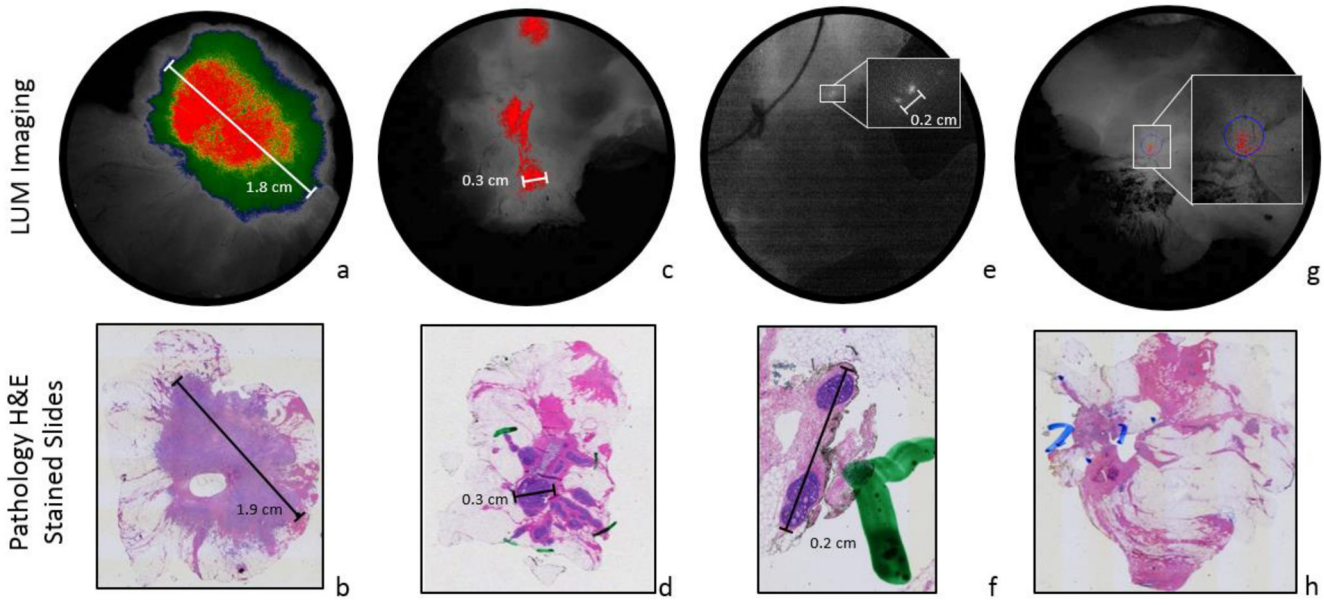

Figure 2:

(a-b) Fluorescent image captured from transected *ex vivo* resected IDC mass from Patient 8. Pathology slide taken from same resected mass; the oval hole within the mass is a processing artifact

(c-d) Fluorescent image captured from transected *ex vivo* resected DCIS specimen in Patient 7. Pathology highlights evidence of 3 mm area of DCIS

(e-f) *Ex vivo* imaging of medial margin from patient 7. Two small fluorescent features appear, enlarged in the inset. Two foci of DCIS appear in the corresponding pathology slide.

(g-h) Lumpectomy transection LUM Image and corresponding H&E stained slide from patient 6. Pathology report defined lesion as invasive mammary carcinoma with mixed ductal and lobular features with DCIS within the invasive carcinoma.

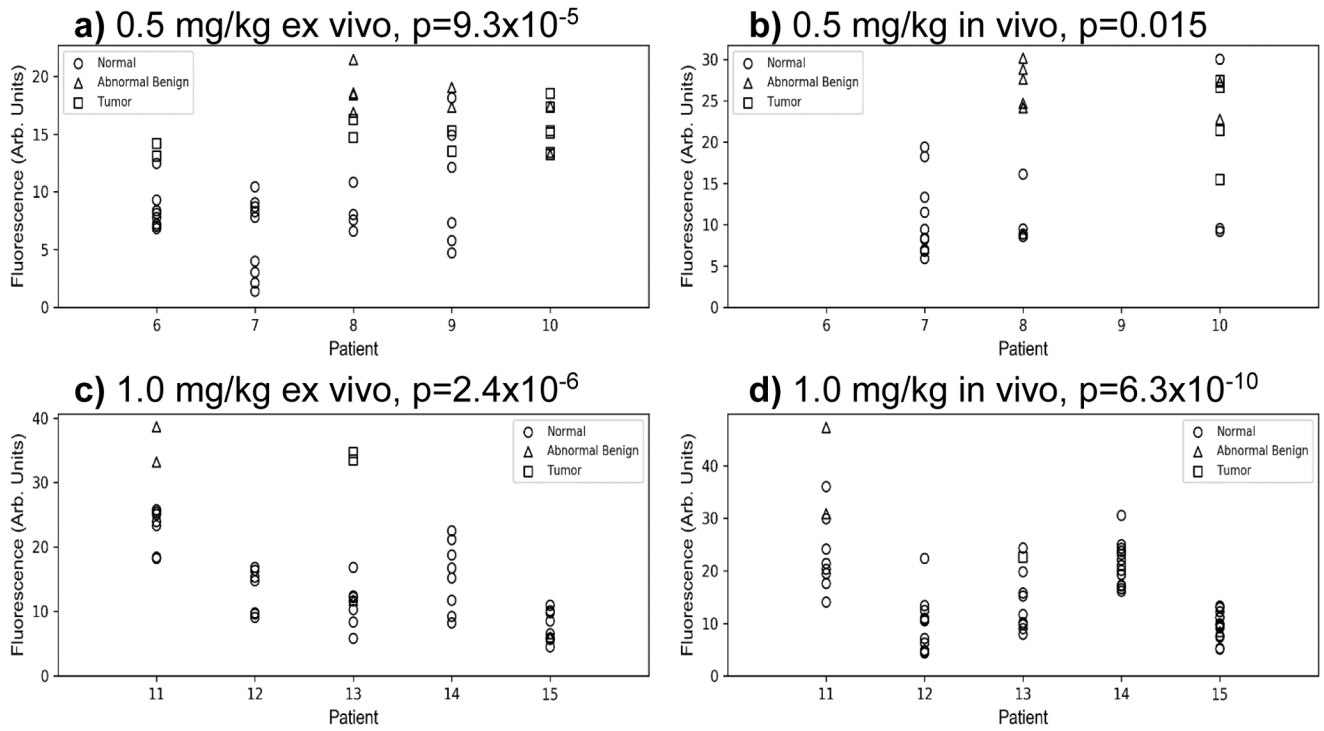

Figure 3:

Ex vivo specimen and in vivo lumpectomy cavity wall fluorescent signal correlated with histopathology. *In* vivo data not available for patients 6 and 9.

Table 1:

Patient, tumor and LUM015 signal characteristics

Patient	LUM015 dose (mg/kg)	Age (years)	Menopause Status	Breast Density	Tumor Histology	Tumor Grade	Largest Tumor Size (cm)	Tumor: Normal (T:N) Signal Ratio
1	0.0	56	Post	Heterogeneously dense	IDC and DCIS (ER+, PR+, HER2+)	2 to 3	0.06	1.42
2	0.0	73	Post	Scattered areas of fibroglandular density	DCIS (ER+, PR+)	1 to 2	5 of 15 blocks	*§*
3	0.0	78	Post	Scattered areas of fibroglandular density	ILC (ER+, PR+, HER2−)	2	1.3	2.83
4	0.0	58	Post	Heterogeneously dense	DCIS (ER+, PR+, HER2−)	2	9 of 21 blocks	*§*
5	0.0	69	Post	Fatty	IDC and DCIS (ER+, PR+, HER2−)	2	1.0	1.9
6	0.5	48	Pre	Heterogeneously dense	Invasive, mixed ductal and lobular features and DCIS (ER+, PR+, HER2−)	1 to 2	1.2	6.48
7	0.5	65	Post	Scattered areas of fibroglandular density	DCIS (ER+, PR+)	2	1.2	3.38
8	0.5	70	Post	Scattered areas of fibroglandular density and heterogeneously dense	IDC and DCIS (ER+, PR+, HER2−)	2	1.9	4.82
9	0.5	56	Post	Heterogeneously dense	IDC and DCIS (ER+, PR+, HER2−)	2	1.8	6.82
10	0.5	65	Post	Heterogeneously dense	IDC and extensive DCIS (ER+, PR+, HER2−)	2 to 3	1.9	1.99
11	1.0	63	Post	Heterogeneously dense	IDC and DCIS (ER+, PR+, HER2−)	1 to 2	0.4	*§*
12	1.0	48	Pre	Heterogeneously dense	IDC and DCIS (ER+, PR−, HER2−)	3	2.5	5.46
13	1.0	56	Post	Heterogeneously dense	IDC, ILC and DCIS (ER+, PR+, HER2−)	2	1.8	6.37
14	1.0	78	Post	Fatty	DCIS (ER+, PR+)	2	2.4	3.22
15	1.0	59	Post	Heterogeneously dense	IDC and extensive DCIS (ER+, PR+, HER2−)	1 to 2	1.4	1.84

*§*Transection data was not collected due to a failure to either obtain fluorescent images or images were taken of sections that did not contain tumor

IDC – invasive ductal cancer, ILC – invasive lobular cancer, DCIS – ductal carcinoma in situ, ER – estrogen receptor, PR – progesterone receptor, HER2 – human epidermal growth factor receptor 2

Table 2:

LUM015 Tumor:Normal (T:N) signal ratio by LUM015 dose and tumor histology.

Injection and Tumor Stratification	Average LUM015 (T:N) Signal Ratio
No LUM015 Injection (n=3)	2.05
0.5mg/kg LUM015 Injection (n=5)	4.70
1.0mg/kg LUM015 Injection (n=4)	4.22
Pure DCIS or extensive DCIS * w/either injection level (n=4)	2.61
Invasive ± DCIS w/either injection level (n=5)	5.99

* Extensive DCIS defined as DCIS within and beyond the area an invasive tumor mass.