Tumor-specific uptake of fluorescent bevacizumab-IRDye800CW microdosing in patients with primary breast cancer: a phase I feasibility study

Running Title: Targeted near-infrared fluorescence imaging in breast cancer

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Financial support:

This work was supported by the Center for Translational Molecular Medicine, project MAMMOTH (grant 03O-201), ERC Advanced grant OnQView and by the Dutch Cancer Society KWF by a research fellowship to SGE.

Conflict of interest:

VN is co-founder of SurgVision BV (Heerenveen, the Netherlands), GMvD and VN are members of the scientific board of SurgVision BV. GMvD, WBN and PJvD have received an unrestricted research grant from SurgVision BV for the development en evaluation of image-guided fluorescence technology and tracers, not related to this study. All consumables (i.e. bevacizumab and IRDye800CW) were commercially obtained.

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Manuscript Information

Word count: 4,698 words

Translational relevance statement: 144 words

Total number of figures: 5 figures

Total number of tables: 1 table

Supplemental Material and Methods / Figures: 7 figures, 1 movie and 1 file

TRANSLATIONAL RELEVANCE

Tumor-free surgical margins are critical in breast conserving surgery (BCS), as local recurrence rates increase with positive margins. Molecular imaging is a promising strategy for visualizing and quantifying tumor-specific molecular characteristics, and potentially improve breast cancer care in terms of detection, characterization and (non-)surgical treatment strategies. A potential target for molecular imaging is vascular endothelial growth factor (VEGF)-A, involved in tumor angiogenesis. Our study shows that systemic administration of the fluorescent bevacizumab-IRDye800CW tracer is safe in *ex vivo* breast cancer guidance and confirms (tumor-)margin uptake providing a novel framework for systematic evaluation and validation of fluorescent tracers in image-guided surgery and drug development. As neo-angiogenesis is a universal tumor marker, other tumor types like colorectal and esophageal cancer might benefit from fluorescence-guided molecular endoscopy using bevacizumab-IRDye800CW. This approach is of interest for surgical guidance, but also for diagnostic purposes, drug development and treatment monitoring.

ABSTRACT

Purpose: to provide proof of principle of safety, breast tumor-specific uptake and positive tumor margin assessment of the systemically administered near-infrared fluorescent (NIRF) tracer bevacizumab-IRDye800CW targeting vascular endothelial growth factor (VEGF)-A in breast cancer patients. *Experimental Design:* Twenty patients with primary invasive breast cancer eligible for primary surgery received 4.5 mg bevacizumab-IRDye800CW as intravenous bolus injection. Safety aspects were assessed as well as tracer uptake and tumor delineation during surgery and *ex vivo* in surgical specimens using an optical imaging system. *Ex vivo* multiplexed histopathology analyses were performed for evaluation of biodistribution of tracer uptake and co-registration of tumor tissue and healthy tissue. *Results:* None of the patients experienced adverse events. Tracer levels in primary tumor tissue were higher compared to those in the tumor margin ($P < 0.05$) and healthy tissue ($P < 0.0001$). VEGF-A tumor levels also correlated with tracer levels ($r = 0.63$, $P < 0.0002$). All but one tumor showed specific tracer uptake. Two out of 20 surgically excised lumps contained microscopic positive margins detected *ex vivo* by fluorescent macro- and microscopy and confirmed at the cellular level. *Conclusions:* Our study shows that systemic administration of the bevacizumab-IRDye800CW tracer is safe for breast cancer guidance and confirms tumor and tumor-margin uptake as evaluated by a systematic validation methodology. The findings are a step towards a phase II dose-finding study aimed at *in vivo* margin assessment and point to a novel drug assessment tool that provides a detailed picture of drug distribution in tumor tissue.

INTRODUCTION

Breast cancer is the second most common cancer, with 522,000 deaths globally in 2012 and a rising incidence (*1*). Surgery is one of the three cornerstones of primary invasive breast cancer treatment, the other two being radiotherapy and systemic therapy. Tumor-free surgical margins are critical in breast conserving surgery (BCS), as local recurrence rates increase with positive resection margins (*2-5*). Therefore, patients with positive resection margins are often re-operated, causing higher surgical risks, poorer cosmetic results, psychological and physical burden and higher healthcare costs. Currently, pre-operative imaging and tactile information are used during breast surgery to determine the size, localization and extent of the area that has to be removed. However, the efficacy of this approach is poor, with positive margin rates of 20% to 40% being reported worldwide (*6*).

Molecular imaging is a promising strategy to improve this efficacy; it can be used to visualize and quantify tumor-specific molecular characteristics, and could potentially improve breast cancer care in terms of detection, characterization and surgical and non-surgical management strategies. A potential target for molecular imaging in breast cancer is vascular endothelial growth factor (VEGF)-A, a soluble dimeric glycoprotein that is involved in tumor angiogenesis (*7,8*). VEGF-A is frequently overexpressed in breast cancers with an expression rate of ~73% compared to normal breast tissue (*9*). It can therefore serve as a more generic tracer target (*10-11*) without pre-selection of patients compared to other potential targets such as human epidermal growth factor receptor 2 (HER2), which is overexpressed in only 10 to 20% of primary breast cancers *(12-14*).

The registered humanized monoclonal antibody bevacizumab not only neutralizes all VEGF-A isoforms, but can also be used as a radiolabeled imaging agent in combination with single photon emission computed tomography (SPECT) and positron emission tomography (PET) (*15*). Successful and specific imaging has been performed in patients with melanoma, renal cell cancer, neuroendocrine tumors and breast cancer using ^{111}In- and ^{89}Zr-radiolabeled bevacizumab (*11,15-19*). In these studies, a systemic total microdose of 4.5 mg labeled bevacizumab, i.e. ≤ 30 nmol adhering to the definition of microdosing for proteins according to FDA/EMEA guidelines (*20*) and as described separately for proteins by Kummar et al *(21),* was used which is sub-therapeutic compared to the therapeutic dose of 5-15 mg/kg bodyweight (*22-24*). In a recent PET imaging study with ^{89}Zr-bevacizumab in 23 primary breast cancer patients, 25 of 26 tumors (96%) were visualized by PET 4 days after ^{89}Zr-bevacizumab tracer injection with tumor-to-normal tissue ratios of 1.4-10.3 (*19*). These results prompted us to design an optical imaging study based on the microdosing concept of using a therapeutic antibody as targeting moiety in patients undergoing breast cancer surgery.

Recently, optical fluorescence imaging has become suitable for clinical translation due to its favorable characteristics such as absence of ionizing radiation, inherently low-cost technology, and its possibilities for real-time, intra- and post-operative imaging. Two phase I feasibility studies in respectively patients with colorectal cancer imaged with a chimeric fluoresceine conjugated Carcino Embryonic Antigen (CEA) targeted antibody (*25*) and ovarian cancer patients using folate-fluorescein isothiocyanate (FITC, light emission at 400-650 nm) targeting the folate receptor-alpha, demonstrated the great potential of optical imaging during surgery using a clinical prototype camera (*26*). However, clinical implementation studies have been obstructed by characteristics such as the limited penetration depth of fluorescent dyes such as fluorescein with

emission in the visible light spectrum, the negative effects of optical properties such as absorption, scattering and the autofluorescence of tissue in the visible spectrum. To reduce background fluorescence and autofluorescence and increase tissue penetration, near infrared fluorescent (NIRF) dyes must be used that have excitation wavelengths between 700-900 nm (*27*). More recently, Burggraaf et al demonstrated c-met targeted endoscopy fluorescence imaging in humans using a fluorescent dye of 650 nm (*28*), whereas Rosenthal et al have demonstrated the feasibility of using the therapeutic monoclonal antibody cetuximab targeting Epidermal Growth Factor Receptor (EGFR), as the targeting moiety of a NIRF tracer in patients with head- and neck cancer (*29*). Additionally, the clinical application of a protease-activatable tracer has been reported in patients with soft tissue sarcoma and breast cancer (*30*).

Using bevacizumab conjugated to the NIRF dye IRDye800CW (peak absorption 778 nm, peak emission 795 nm), we decided to determine if this approach could be used for intraoperative guidance in breast cancer surgery, combined with a novel systematic analytic methodology for ex vivo evaluation of tumor-specificity and microdistribution. Bevacizumab-IRDye800CW showed high levels of accumulation in human breast cancer bearing mouse tumors *(31),* leading to Good Manufacturing Practice (GMP) production of clinical grade bevacizumab-IRDye800CW for human use *(32).* In conjunction with the progress in the development of NIR intra-operative optical imaging systems for clinical applications, we initiated this first in-human clinical study of clinical grade bevacizumab-IRDye800CW in breast cancer patients.

The primary aims of our feasibility study were to provide proof of principle of safety, tumor-specific uptake and tumor margin assessment of the intravenously administered NIRF microdose tracer bevacizumab-IRDye800CW, targeting VEGF-A in patients with primary invasive breast cancer, as validated *ex vivo* by multiplex advanced pathology imaging.

MATERIALS AND METHODS

Trial Design

The study was a two center, first in human, two-stage, non-randomized, non-blinded, prospective, feasibility study in patients with histologically proven breast cancer scheduled for surgery (registered at www.clinicaltrials.gov, identifier NCT01508572). Primary endpoints were the occurrence of serious adverse events (SAE, adverse events were classified according to the National Cancer Institute Common Terminology Criteria for Adverse Events (Version 4.0)) and accumulation of bevacizumab-IRDye800CW in breast cancer tissue and surrounding tissue in surgical specimen by fluorescence macroscopy and microscopy. Additional information is provided in Supplementary Materials and Methods, section "*Trial Design*". The Institutional Review Board (IRB) of the University Medical Center Groningen (UMCG) approved the study, with local agreement of the University Medical Center Utrecht (UMCU). All patients gave written informed consent.

Bevacizumab-IRDye800CW Preparation and Injection

Clinical grade bevacizumab-IRDye800CW was produced in the GMP facility of the UMCG by labeling bevacizumab (Roche AG) and IRDye800CW-NHS (LI-COR Biosciences Inc.) under regulated conditions as described previously (*31*). The molecular weight of the protein bevacizumab is 149 kDa. The fluorescent dye has a molecular weight of 1,166 kDa. With an average conjugation ratio of 1:4 protein:IRDye800CW, the total molecular weight is 153,7 kDa. This means for our 4.5 mg tracer dose bevacizumab-IRDye800CW 26 nmol of bevacizumab-IRDye800CW, adhering to the FDA/EMA regulations of microdosing for proteins and according to the Task Force on Methodology for the Development of Innovative Cancer Therapies

(MDICT) (*20,21*). Additional information is provided in the Supplementary Material and Methods, section "*Bevacizumab-IRDye800CW Preparation and Injection*".

Surgical Procedures and Specimen Handling

Patients underwent mastectomy or lumpectomy with or without a sentinel lymph node (SLN) procedure or axillary LN dissection, according to standard-of-care procedures and guidelines applicable for breast cancer in The Netherlands *(33*). During the study period, the positive margin rate for the University Medical Center Groningen was 7.8% and for the University Medical Center it was 5.2%.

In short, the SLN procedure was carried out by peri-tumoral injection of Technetium-99m nano-colloid followed by lymph scintigraphy after 2-3 hours. During surgery a hand-held gamma counter was used to detect the radioactive signal from the SLN. After the first patient, the protocol was amended to omit patent blue V (Guerbet Asia Pacific) for visualization of the SLN if possible, as this dye interfered with the fluorescent signal after systemic microdosing 4.5 mg bevacizumab-IRDye800CW in this patient during surgery.

During surgery, images were recorded at several pre-defined time points during removal of the tumor and SLN with the optical imaging system (for further details see Supplementary Material, section "*Optical Imaging System*"). A baseline image was recorded before incision of the breast, followed by imaging of the tumor, before removal of the tumor (lumpectomy or mastectomy) at a distance suitable for the fluorescent signal (on average 10-15 cm above the operating field), but not interfering with the sterile field of surgery. Using the optical imaging system prior to incision at the maximum field-of-view (FOV), the presence of a fluorescence signal was determined either in the tumor or axillary region. Identified (S)LNs were imaged prior to excision. After removal of the tumor and SLN, the surgical field was inspected again for remaining fluorescent signals. The surgical approach could only be adapted to the intraoperative

findings if this would not have a negative impact on the actual outcome of the surgical procedure as judged by the attending breast cancer surgeon (authors LJ, JdV and AJW) in terms of cosmetic outcome and impaired wound healing. Subsequently, excised tumor tissue and SLN(s) were imaged *ex vivo* off-table directly after removal. Next, the surgical specimen was processed by the pathologist for *ex vivo* analysis of tumor samples according to standard procedures and including imaging of the specimen as described in the subsection 'Ex vivo analyses of tumor samples'. For all tumors besides determination of size, extent, presence of *in situ* carcinoma, expression of estrogen receptor (ER), progesterone receptor (PR) and HER2 histological grading and typing according to the modified Bloom and Richardson and WHO guidelines was also performed according to standard clinical practice.

Follow up

Adverse events occurring through approximately 2 weeks after surgery were recorded as spontaneously reported by patients or at an outpatient visit.

Ex vivo analyses of tumor samples

The following subsections are provided in the Supplementary Material and Methods, section "*Ex vivo analyses of tumor samples*": i) Imaging fresh surgical specimen, ii) Imaging FFPE blocks and slides, iii) Bevacizumab-IRDye800CW *(34)* and VEGF-A quantification, iv) Immunohistochemistry, v) Fluorescence microscopy, vi) Multiplex Advanced Pathology Imaging (MAPI) methodology.

Statistical Analysis

Details on statistical analysis and power calculations for detecting potentially clinically relevant bevacizumab-IRDye800CW breast accumulation in patient with primary breast cancer is provided in Supplemental Material, section "*Statistical Analysis*".

RESULTS

Patient Characteristics

Between March 2012 and August 2014, we enrolled 20 patients with breast cancer (including one male patient) in the study. Patient and tumor characteristics are summarized in Table 1. Most patients had an invasive ductal carcinoma (n=17, 85%), and three had an invasive ductulolobular carcinoma. Tumor size determined by pathology ranged from 6 mm to 38 mm (median 20 mm) in diameter. Histological analyses after surgery of the tumor showed Bloom-Richardson-Elston histology grade 1 in 6 tumors, grade 2 in 10 tumors and grade 3 in 4 tumors. Estrogen receptor (ER) status was positive in 18 patients (90%), progesterone receptor (PR) status was positive in 14 patients (70%), and HER2 expression scores were negative in 13 patients (65%), 1+ in five patients, 2+ in one patient and 3+ in one patient. In three patients, all the excised tissue was required for standard histological examination due to the small tumor sizes of 6 mm. Two patients (10%) undergoing BCS through a lumpectomy had a positive resection margin on standard histopathological examination, 18 none.

No adverse events related to the tracer injection occurred in any of the patients, nor aberrations in hematology or blood chemistry levels. In none of the patients tumor recurrence occurred during follow-up after surgery.

Intra-operative Imaging

Specimens from two patients were identified with a microscopic irradical resection (i.e. the positive margin) upon histopathological analysis (Fig. 1 and Supplemental. Fig. S1). In both patients a fluorescent signal was detectable at the positive resection margin of the excised lump (Fig. 1A-C), although not visible during surgery, but clearly visible on the back table during the surgical procedure and after bread-loaf slicing (Fig. 1D-F). The paraffin block (Fig. 1G - 1I) also showed a clear positive signal at the tumor site. Hematoxylin and eosin stain (H/E, Fig. 1J)

findings were corroborated by fluorescence flatbed scanning (Fig. 1K) and overlay image (Fig. 1L). This confirmed the presence of bevacizumab-IRDye800CW in the tumor area and the designated positive margin in both patients. Supplemental Fig. S1 shows the surgical specimen from the second patient with a positive margin, visualized with our standard operating procedure for *ex vivo* processing. Fluorescence imaging shows strong signals at the vicinity of the margin in the bread-loaf slices (Fig. S1, panel A), which was confirmed by NIR optical imaging of paraffin blocks (Fig. S1, panel B, tumor is demarcated by red segmentation), H/E staining (Fig. S1, panel C), and fluorescence flatbed scanning (Fig. S1, panel D and E). With one exception, we detected a fluorescent signal in the excised specimens of all patients who were injected with bevacizumab-IRDye800CW. In the first patient, a male with breast cancer, in whom we could not detect a fluorescent signal *ex vivo*, a fluorescent signal prior to incision in the breast was visible but disappeared upon injection of patent blue (Patent Blue V Sodium Guerbet 2.5% solution for injection, 1 mL peritumoral injection) as part of the sentinel lymph node (SLN) procedure. For the remaining 19 patients, patent blue was omitted within the SLN protocol and accordingly approved by the local IRB. It was concluded that the disappearance of the NIR fluorescence originating from the systemic injection of a microdose of bevacizumab-IRDye800CW in the tissue specimen was due to complete absorbance by the abundant peritumoral injection of patent blue and therefore subsequently omitted. Excised specimens were imaged by flatbed scanning of the paraffin blocks. Patients who were not injected with bevacizumab-IRDye800CW (negative controls) did not show fluorescent signals above background levels in tumor areas (Supplemental Fig. S2). In one patient, the skin was very fluorescent and in one patient a fibroadenoma adjacent to the tumor showed a clear fluorescent signal. In all patients a Standard Operating Procedure for *Ex Vivo* Processing of Surgical Specimen was applied (Fig. 2).

Bevacizumab-IRDye800CW Blood and Tissue Concentrations

The whole blood concentrations of bevacizumab-IRDye800CW decreased during 14 days after injection (Fig. 3A). The bevacizumab-IRDye800CW concentration in tumor tissue was higher compared to the margin ($P < 0.05$) or surrounding non-cancerous tissue ($P < 0.0001$) (Fig. 3B). VEGF-A levels differed between tumor and surrounding tissue ($P < 0.001$) and between margin and surrounding tissue, ($P < 0.05$) but not between tumor and margin (not significant) (Fig. 3C). Bevacizumab-IRDye800CW and VEGF-A concentrations by ELISA correlated in the tumor area (r = 0.63, $P = 0.0002$) (panel D), but not significantly at the margin or surrounding non-cancerous tissue (Fig. 3D-F). Additional SDS-PAGE analysis of tumor lysates of three patients, confirmed the intactness of the bevacizumab-IRDye800CW tracer within the tumor.

Fluorescence Imaging and Tumor Margins

To compare macroscopic fluorescence imaging with tumor margins, two methods were used: tumor area assessment by defining four tumor margin zones distant from the tumor and by segmentation (Fig. 4). After flatbed scanning (Fig. 4, panel A) and H/E staining of the paraffin block (Fig. 4, panel D), four margin zones of 5 x 20 mm were defined (tumor area, 0.5 cm, 1.0 cm, 1.5 cm) and scanned (Fig. 4, panel B), after which the mean fluorescence intensity (MFI) was determined (Fig. 4C). The MFI of the tumor area differed from the other three tumor margin zones (tumor vs. 0.5 cm, tumor vs. 1.0 cm, tumor vs. 1.5 cm, all $P < 0.0001$).

Similarly, segmentation for separating tumor, stroma and fat was performed on the excised specimen after H/E staining (Fig. 4E, F and G). Next, the segmented areas were superimposed on the fluorescence flatbed scans (Fig. 4E), and the MFI was calculated (Fig. 4H). Tumor segmented areas had a higher MFI compared to stroma ($P < 0.0001$) and fat ($P < 0.0001$), which also translated into a significant difference in target-to-background ratios for tumor/stroma versus tumor/fat ($P < 0.001$) (Supplemental Fig. S2). In two patients with a positive margin, the

margin was fluorescent, while in the remaining 18 patients with a negative margin there was also no fluorescence in the resection margin. In 90% of all patients there was adjacent / complete overlap of bevacizumab-IRDye800CW and VEGF-A immunohistochemistry staining and in 10% no overlap (Fig. 5).

Multiplex Advanced Pathology Imaging (MAPI)

For a more detailed analysis of the distribution of bevacizumab-IRDye800CW in tumor tissue, a standardized operating procedure (SOP) for macro- and microscopic mapping was performed at a macroscopic level as depicted in Fig. 2 and at the cellular level in Supplemental Fig. S3 – S5.

H/E staining of 4 μm tissue slides (Fig. S3, panel A and B) was compared to fluorescence scanning (Fig. S3, panel C and D). In a superimposed image, both modalities were compared on a macroscopic and microscopic level (Fig. S3, panel E and F). Fluorescence signal was clearly identified in a tumor sprout surrounded by blood vessels and collagen-rich stroma (rectangle, Fig. S3, panel B, D and F). NIR fluorescence microscopy compared to co-localization of H/E staining for tumor (margin) specificity clearly indicated tumor-specific staining of bevacizumab-IRDye800CW (Fig. S3, panel G and H).

For qualitative co-localization of bevacizumab-IRDye800CW with other biomarkers such as VEGF-A expression, CD34 staining for microvessel density and presence of collagen, MAPI was used (Supplemental Movie S1). Fluorescent bevacizumab-IRDye800CW scans were pseudocolored green, whereas hematoxylin-DAB/VEGF-A staining color intensities were color deconvoluted into pseudocolor red and superimposed with the pseudocolor green bevacizumab-IRDye800CW scans.

Supplemental Fig. S4 shows the co-localization of bevacizumab-IRDye800CW, H/E, VEGF-A, collagen and CD34 in breast cancer and a satellite lesion. A clear co-localization of

fluorescence intensities as determined by fluorescence imaging (Fig. S4, panel A-D) is present within the tumor area (dotted lines). This was confirmed with H/E staining (Fig. S4, panel E), segmentation (Fig. S4, panel F), pseudocolor green bevacizumab-IRDye800CW flatbed scan (Fig. S4, panel G) and superimposed H/E with pseudocolor green (Fig. S4, panel H). Furthermore, co-localization of fluorescence and VEGF-A staining (Fig. S4, panel I), collagen (Fig. S4, panel M) and CD34 (Fig. S4, panel Q) could be visualized as such. Additional data is provided for co-localization in ductal carcinoma in situ (DCIS) in Supplemental Fig. S5.

In 18/20 patients, lymph nodes were excised. Only three patients had a tumor positive sentinel lymph node, 13 patients had a negative sentinel lymph node. Two additional patients had lymph nodes with macrometastases: one of them had clinical suspicious macrometastatic lymph nodes, confirmed by ultrasound guided biopsy and axillary lymph node dissection. Only in this patient we could even clearly identify fluorescence activity intraoperatively within the lymph node dissection specimen in situ, confirmed by ex vivo fluorescence and histopathological analysis (Supplemental Fig. S6). There was no difference in VEGF-A IHC staining between tumor-positive lymph nodes and negative lymph nodes (n=104 lymph node tissue blocks). Co-localization of NIR fluorescence showed mainly fluorescence surrounding the tumor cells within a lymph node (Supplemental Fig. S6), whereas in a tumor-negative lymph node it was mainly centrally located in the lymph node (Supplemental Fig. S7).

DISCUSSION

In this first in-human study using a NIRF antibody-based tracer in a microdose regimen, administration of bevacizumab-IRDye800CW was safe with sufficient tumor-specific tracer uptake for margin assessment in primary breast cancer tissue. A novel framework of systematic *ex vivo* macroscopic imaging and fluorescent image analyses of excised specimen, fresh tissue slices, paraffin blocks and tissue slides showed tumor-specific tracer uptake, thus clearly distinguishing the tumor margins within normal healthy breast tissue. This framework provides a novel tool for evaluation and validation of fluorescent tracers in image-guided surgery. NIR light has low tissue absorption characteristics, mainly caused by hemoglobin, and low autofluorescence properties compared to fluorescent dyes in the visible light (*27*). In BCS, a negative microscopic resection margin is of great value in order to reduce a re-operation or the risk of recurrent disease *(35)*, even after adjuvant radiotherapy. A NIRF tumor-specific tracer is therefore the most suitable in terms of sensitivity and specificity whether (microscopic) residual tissue is present and should accordingly be excised.

Most prior optical imaging efforts in breast cancer patients used the non-targeted NIRF tracer ICG, which binds to plasma proteins. This has been tested extensively for lymphatic mapping in breast cancer (*36-40*). In breast cancer patients, ICG had SLN identification rates comparable to standard-of-care radiotracers and blue dyes (*40*). However, ICG is unsuitable due to the limiting formulation and quenching characteristics of the dye for simple and straightforward conjugation to targeting moieties, like antibodies, nanobodies or small peptides. This precludes its use for tumor-specific targeting by NIRF imaging, as in the present study. At this point, two clinical landmark studies have been published on the use of fluorescent targeted antibodies. One study targeting Carcino Embryonic Antigen (CEA) in colorectal cancer *(25)*, and

more recently Epidermal Growth Factor Receptor (EGFR) in head- and neck cancer *(29).* Several approaches have been used showcasing the potential of fluorescence imaging in humans, varying from single-dose to dose-escalation designs differing from our micro-dosing approach. In particular, the *ex vivo* validation steps for visualization of tumor microdistribution of the NIR fluorescent tracer and its relationship to histological immunohistochemical parameters provides a framework which was not reported earlier in previous clinical studies in for example fluorescein-conjugated CEA-targeted imaging in colorectal cancer (*25*), folate receptor-α imaging in ovarian cancer (*26*), the study of Rosenthal et al using cetuximab-IRDye800CW in head- and neck cancer (*29*) and a protease activatable probe in soft tissue sarcoma and breast cancer (*30).* Therefore, the impact of our study is that it provides the necessary framework of evaluation and reporting of future clinical studies of fluorescence image-guided surgery. The *in vivo* targeting characteristics of a GMP fluorescent tracer by applying as a first step the microdosing concept, and thus a low risk of potential adverse events, delivers data on the targeting characteristics of the tracer and subsequently the format for *ex vivo* analyses. If such a study provides the data for tumor specific targeting, then a subsequent dose-finding or diagnostic accuracy study can be carried out with a higher degree of definitive data. As mentioned by Kummar et al (*21*), 'microdosing studies are designed with the objective to establish at the very earliest opportunity – before a large numbers of patients have been accrued and exposed to potential drug-associated toxicity – whether an agent is targeting its biological marker in a tumor, and consequently whether further clinical development is warranted' and thus allows selection of tracer candidates more likely to be developed successfully, but also helps in determination of the dosing-scheme for the subsequent Phase II-III clinical studies.

Although *ex vivo* imaging confirmed tumor specific uptake in our study, the absolute fluorescent signal intensities of the targeted tracer were too low to identify tumor margins *in situ*

during actual surgery, for which several explanations are possible. First, the tracer dose used in this study may have been too low to reach the threshold for detecting tumor-specific signal intra-operatively originating from microscopic irradical tumor margins. Nevertheless, 90% of all patients using microdosing bevacizumab-IRDye800CW showed adjacent or complete overlap with VEGF-A expression measured by IHC. Second, due to the fact that usually surgical incisions in BCS are small, this might imply that insufficient excitation light reaches the surgical cavity or wound bed or vice versa that emitted NIR light originating from the tracer cannot be collected by the optical imaging system. This might be solved by applying a sterile NIR laparoscope or endoscope close within the wound bed. Third, even with a higher dose, improvements in the sensitivity of the camera system, such as correction algorithms and state-of-the-art charge-coupled device (CCD) chips, may be required for intra-operative detection of bevacizumab-IRDye800CW.

By increasing the tracer dose to still sub-therapeutic doses, a signal above the threshold of healthy surrounding autofluorescent signal can be expected, and intra-operative detection of the fluorescent signal in tumor tissue may be feasible. This has recently been shown by Rosenthal et al. up to a maximum dose of 62.5 mg/m^2 of intravenously injected cetuximab-IRDye800CW in patients with head and neck cancer (HNSCC) up (*29*). Similarly, a large clinically proven safe dose range is still available for a bevacizumab NIR tracer. For example, patients treated for colorectal cancer with neo-adjuvant bevacizumab being dosed at 10-15 mg/kg every 3 weeks undergo surgery around 6 weeks after the last bevacizumab dose to avoid wound healing problems. With a half-life of 20 days, the remaining circulating bevacizumab level is around 160 mg at that time. This would translate into a minimum 180 mg flat bevacizumab dose, to be injected 3 days before surgery, without increased risk of impaired wound healing. In future studies, we therefore suggest a dose escalation starting around 10 mg and increasing up to the

potential maximum of 180 mg. The total costs for microdosing and the procedural costs are estimated to be $1800. As VEGF-A is overexpressed in >70% of all patients with breast cancer eligible for breast conserving surgery, pre-selection by applying a ^{89}Zr-bevacizumab PET imaging study seems redundant and imposes in ~30% of the patients an increased radiation risk which does not outweigh the risks associated with an injection of a non-radioactive fluorescent tracer.

In our clinical study we used the NIR optical imaging system not only during surgery, but also post-operatively during pathological examination to image the complete excised specimen, after bread-loaf slicing, in paraffin blocks and on tissue slides. These slice images showed a clear fluorescent signal at the site of the tumor in fresh tissue, as confirmed by fluorescence scanning and microscopy. Fluorescence-guided pathology may thereby assist the pathologist in assessing the important tissue parts or for sensitive sampling, such as tumor margin assessment of a mastectomy or lumpectomy specimens. Moreover, during surgery, the pathologist can immediately report to the surgeon whether all tumor has been excised or if margins show a fluorescent signal, which might indicate the presence of tumor.

By using multiplex advanced pathology imaging (MAPI), a systemically injected bevacizumab-IRDye800CW fluorescent tracer was cross-correlated with the presence of tumor (using H/E), VEGF-A expression (by IHC and ELISA), collagen and microvessel density. Next to visualization of other targets with antibodies, smaller fragments like nanobodies targeting specific tumor markers such as carbonic anhydrase IX, HER2, and carcinoembryonic antigen have been developed more recently for imaging solid tumors. This could also be validated by NIR optical imaging (in situ and ex vivo) and MAPI in future translational clinical studies *(41-43)*. Moreover, the present study has shown that MAPI can be used *ex vivo* to cross-correlate the

fluorescent-labeled therapeutic drug bevacizumab and its tumor-specific targeting and local tumor distribution in humans by using excised specimens. This can be done with both macroscopic and microscopic imaging. This novel co-localization methodology provides a reproducible platform in drug development and subsequent dose-finding studies at the tissue and cellular level for other antibodies (therapeutic or otherwise), nanobodies and small peptides as targeting moiety.

As neo-angiogenesis is a universal tumor marker, other tumor types may also benefit from fluorescence imaging using bevacizumab-IRDye800CW. For example, HNSCC, colorectal and esophageal cancer may be suitable for such imaging, as these tumors are located more superficially, leading to higher fluorescence signals and less negative impact on surrounding tissue (i.e. scattering, penetration issues). Currently, three studies are ongoing to determine feasibility of a fluorescent endoscope attached to the camera system after administration of 4.5 mg bevacizumab-IRDye800CW intravenously to detect rectal cancer, esophageal cancer and pre-malignant and malignant polyps in familial adenomatous polyposis (ClinicalTrials.gov identifiers NCT01972373, NCT02129933 and NCT02113202). This approach is therefore of interest not only for tumor visualization and characterization in intra-operative surgical guidance, but also for diagnostic purposes, drug development and treatment monitoring *(42)*.

In conclusion, this is the first clinical study to demonstrate safety and feasibility of the NIR fluorescent tracer bevacizumab-IRDye800CW for tumor-specific optical imaging in patients with primary breast cancer. Probably, because the administered dose (i.e. microdose of 4.5 mg – 26 nmol) was low, in situ intra-operative tumor margin detection was not possible. However, immediate NIR optical imaging of the excised specimen in patients with a positive margin confirmed reliable margin assessment by fluorescence. Therefore, *ex vivo* imaging was highly feasible and correlated well with VEGF-A quantification and microscopic analyses of the tumor

site of targeting. Microscopic analyses did not show a complete overlay of the fluorescent signal and the VEGF-A staining. This is probably because bevacizumab-IRDye800CW targets the soluble and extracellular matrix-bound splice variant 121 of VEGF-A (*10*,*11*), whereas the applied IHC staining mainly detects intracellular VEGF-A expression. For intra-operative *in situ* imaging purposes, fluorescent intensity values could be optimized using higher tracer doses that are still well below the therapeutic dosing scheme of bevacizumab, such as recently described for cetuximab-IRDye800CW.

We have therefore initiated a subsequent phase II dose-finding study to determine the optimal dose for intra-operative use in patients with primary breast cancer (www.clinicaltrials.gov: NCT02583568).

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Table 1.

Characteristic	N	%	Median (range)
Gender, female	19	95	
Surgery			
Lumpectomy	11	55	
Mastectomy	9	45	
Side tumor			
Right	8	40	
Left	12	60	
Age (y)			65 (46 – 81)
Tumor size (mm)			
Conventional imaging before surgery			
Ultrasound			15 (4 - 40)
MRI (n=1)			28
Pathology			20 (6 - 38)
HISTOLOGY			
DUCTAL CARCINOMA	17	85	
DUCTULOLOBULAR CARCINOMA	3	15	
Bloom-Richardson-Elston grade			
Grade 1	6	30	
Grade 2	10	50	
Grade 3	4	20	
Receptor status			
ER (positive)	18	90	
PR (positive)	14	70	
HER2 (IHC 3+ or 2+ with FISH positive)	1	5	
Ductal carcinoma in situ component (n=15)	2	13.3	
Grade 1	10	66.7	
Grade 2	2	13.3	
Grade 3	1	6.7	
Intracystic papillary carcinoma			
Pathological tumor stage			
T1a	0	0	
T1b	4	20	
T1c	6	30	
T2	10	50	
Pathological nodal stage			
N0	13	65	
N1	7	35	
Positive surgical margin	2	10	

Legends of Tables and Figures

Table 1. **Patient demographic and pathological characteristics**. Tumor grade according to the Bloom–Richardson–Elston system (grade 1, 2, or 3).

Figure 1. **Optical Imaging of Positive Tumor Margin.** In one of the two patients (see also Supplemental Fig. S1) with a positive margin (tumor characteristics: lobular carcinoma, diameter 2.3 cm, Bloom-Richardson-Elston grade 1, mitotic activity index (MAI) 2, estrogen receptor positive, progesterone receptor negative, HER2 negative), the bevacizumab-IRDye800CW tracer in the positive margin (black and white arrows) could be detected *ex vivo* by optical imaging of the excised lump (panel A white-light, panel B fluorescence, panel C overlay with pseudocolor). Bread-loaf slicing (panel D-F) and the paraffin block (panel G-I) is shown for the corresponding fluorescent signal. Hematoxylin/eosin (H/E) stain (panel J), fluorescence flatbed scanning (panel K) and image overlay of panel J and K (panel L) is shown for the 4 μm slide (* = skin with suture in place, ** = satellite tumor foci, red/white/black dashed line = tumor outline, white/black arrow = positive tumor margin).

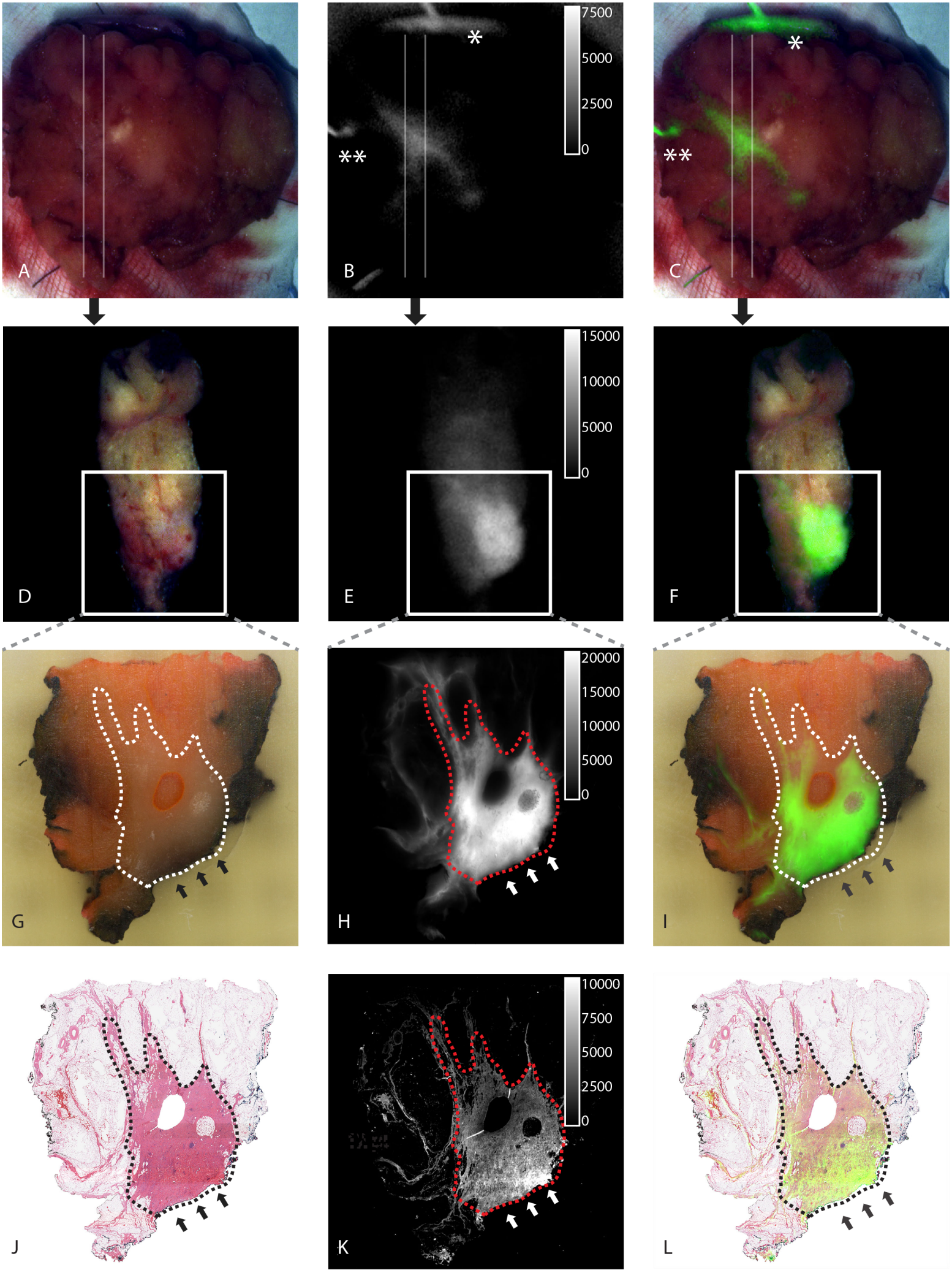
Figure 2. **Standard Operating Procedure for *Ex Vivo* Processing of Surgical Specimen.** Upon excision during surgery, the entire fresh specimen was cut into bread-loaf slices and imaged by the optical imaging system in overlay images of white light and fluorescence mode (panel A, fluorescence = green pseudocolor, asterisk = tumor). Next, tissue was embedded into paraffin blocks and overlay images of white light and fluorescence were taken (panel B, red = tumor border, green = pseudocolor for fluorescence). In panel C, hematoxylin/eosin (H/E) staining is shown of slides from the same paraffin blocks (red = tumor localization) for co-localization purposes with the fluorescence images. Prior to H/E staining, the paraffin blocks were scanned on a flatbed scanner (panel D, depicted in mean fluorescence intensity (MFI)), and also depicted as a Manhattan intensity graph in panel E.

Figure 3. ***Ex Vivo* Quantification of Bevacizumab-IRDye800CW and VEGF-A in Whole Blood and Tissue.** Blood concentration levels (mean ± standard deviation) of bevacizumab-IRDye800CW (ng/ml) decreased 14 days after injection of the tracer (panel A). In tumor tissue biopsies there was a higher concentration of bevacizumab-IRDye800CW (ng/mL/mg of weight biopsy tissue) compared to the tumor margin (* = $P < 0.05$) and surrounding (non-cancerous) tissue (**** = $P < 0.0001$) (panel B). VEGF-A (pg/mg) concentrations differed significantly between tumor vs. surrounding tissue (*** = $P < 0.001$) and margin vs. surrounding tissue (* = $P < 0.05$), but not between tumor area and margin (ns) (panel C). Bevacizumab-IRDye800CW and VEGF-A correlated in the tumor area ($r = 0.63$, $P = 0.0002$) (panel D), which was not apparent at the margin (panel E), or in surrounding non-cancerous tissue (panel F).

Figure 4. **Macroscopic Fluorescence Tumor Margin Assessment.** Paraffin blocks were inked for margin assessment as standard of care and subsequently imaged by macroscopic fluorescence imaging, followed by H/E staining (panel A). By defining four tumor margin zones distant from the tumor (i.e. tumor area, 0.5 cm, 1.0 cm and 1.5 cm, panel B), the mean fluorescence intensity (MFI) was calculated (panel C). MFI of the tumor site was higher than in the tumor margin zones (*** = $P < 0.0001$). **Macroscopic Segmentation of Fluorescence Tissue Localization.** Paraffin blocks were scanned (panel A), stained for hematoxylin/eosin (H/E) (panel D), and subsequently segmented (panel E and F). For each tissue type the region-of-interest (ROI) was delineated (panel G, tumor [RED], stroma [GREEN] and fat [BLUE]). Per ROI, mean fluorescence intensity (MFI) was calculated and compared (panel F). Tumor tissue had a higher MFI compared to stroma and fat (*** = $P < 0.001$).

Figure 5. **Co-localization of Vascular Endothelial Growth Factor-A (VEGF-A) and Bevacizumab-IRDye800CW.** Color deconvolution (DAB color layers were converted to red-black images) of immunohistochemistry staining for VEGF expression is depicted in red. Near-infrared fluorescence of bevacizumab-IRDye800CW is depicted in green. Between VEGF-A and bevacizumab-IRDye800CW localization there was in 90% of the patients adjacent / complete overlap (18/20 patients) and no overlap in 10% of the patients (2/20 patients).

Figure 1


A
B
7500
5000
2500
0
*
**
C
*
**
D
E
15000
10000
5000
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F
G
H
20000
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I
J
K
10000
7500
5000
2500
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L

Figure 2

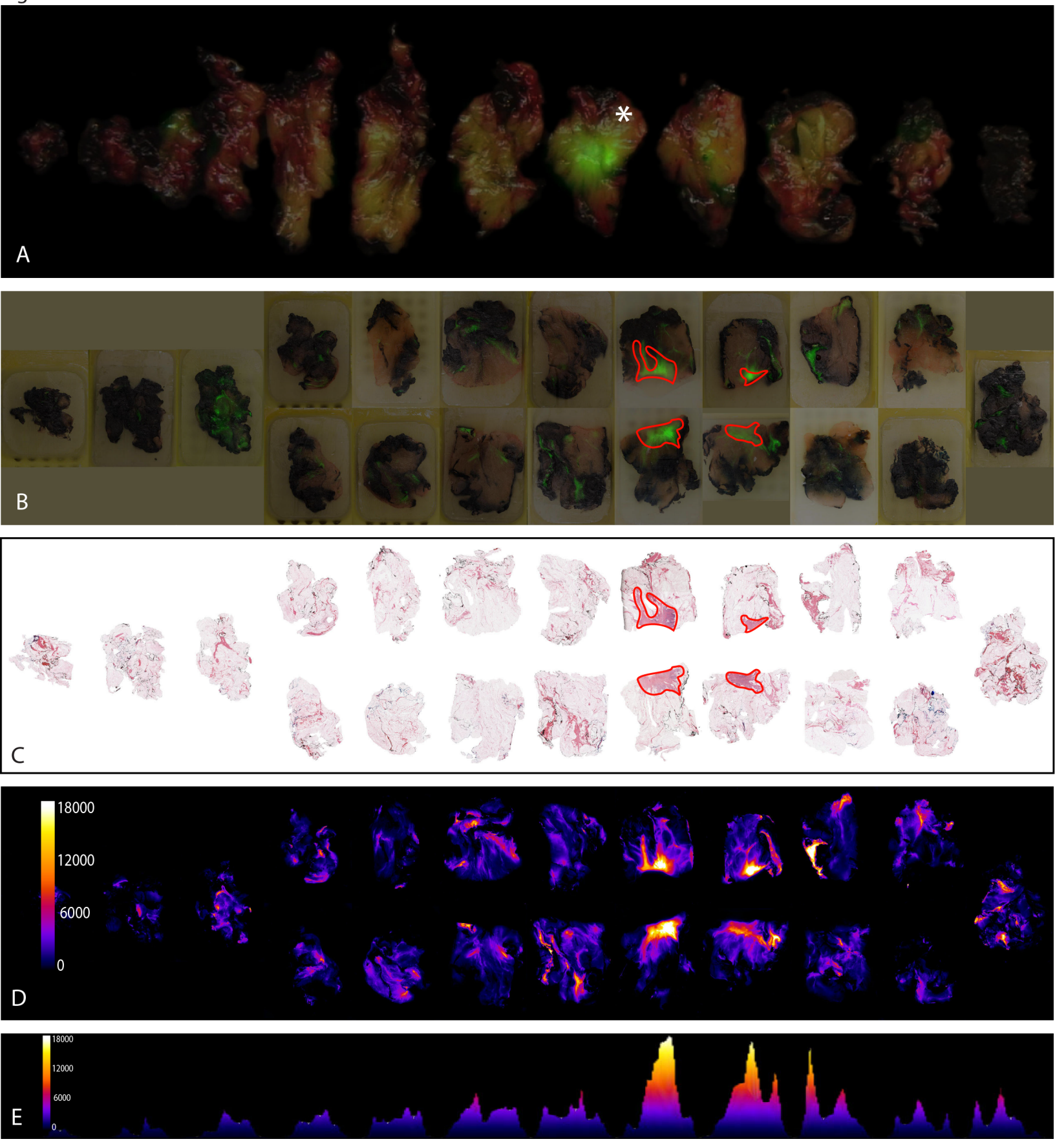


Figure 3

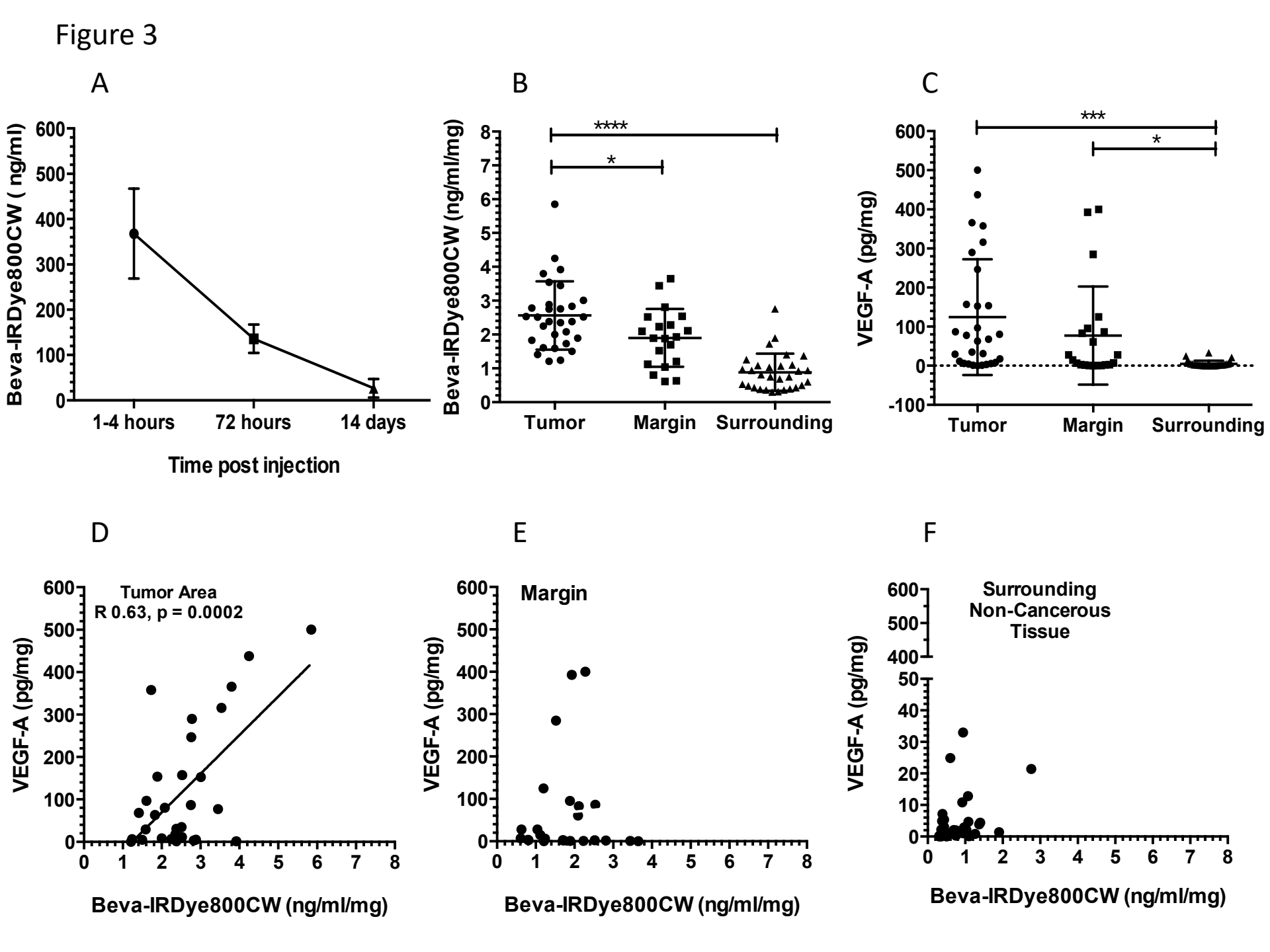
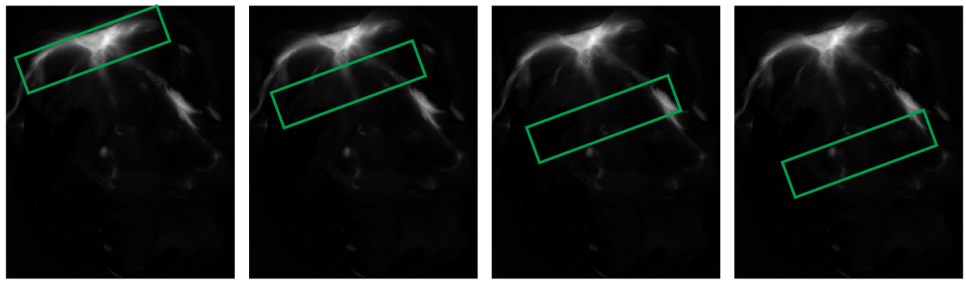
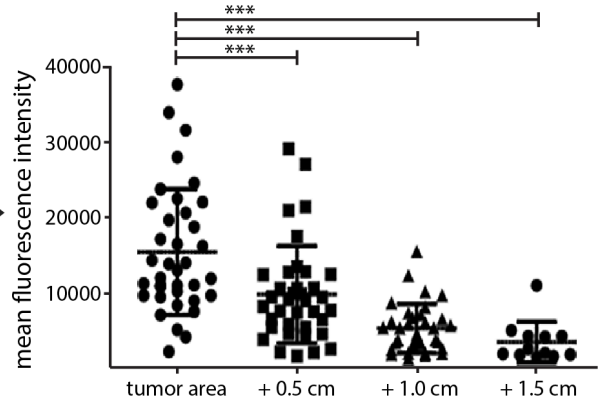

A
Beva-IRDye800CW (ng/ml)
600
500
400
300
200
100
0
1-4 hours
72 hours
14 days
Time post injection
B
Beva-IRDye800CW (ng/ml/mg)
8
7
6
5
4
3
2
1
0
****
*
Tumor
Margin
Surrounding
C
VEGF-A (pg/mg)
600
500
400
300
200
100
0
-100
***
*
Tumor
Margin
Surrounding
D
Tumor Area
R 0.63, p = 0.0002
VEGF-A (pg/mg)
Beva-IRDye800CW (ng/ml/mg)
E
Margin
VEGF-A (pg/mg)
Beva-IRDye800CW (ng/ml/mg)
F
Surrounding
Non-Cancerous
Tissue
VEGF-A (pg/mg)
Beva-IRDye800CW (ng/ml/mg)

Figure 4

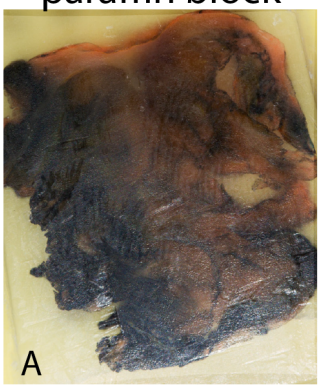


B



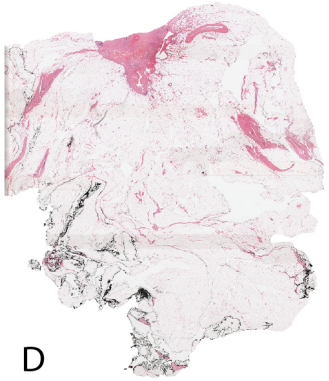
C

paraffin block



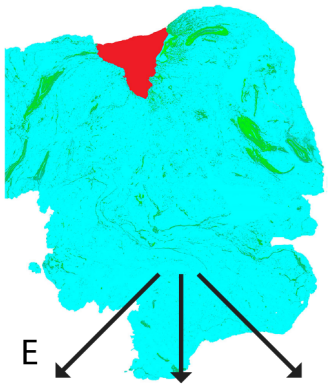
A

H/E slide

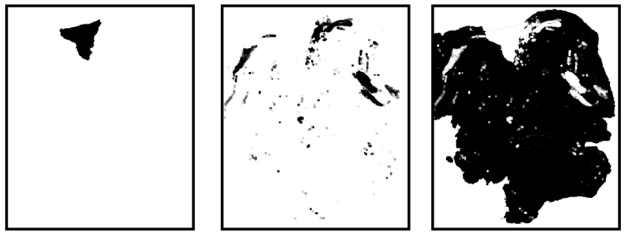


D

segmentation

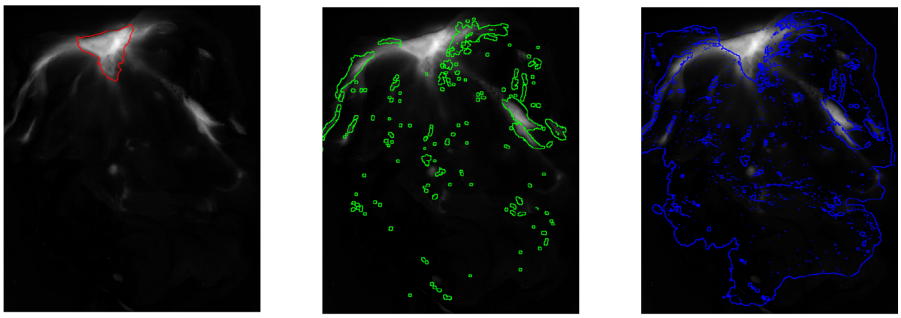


E

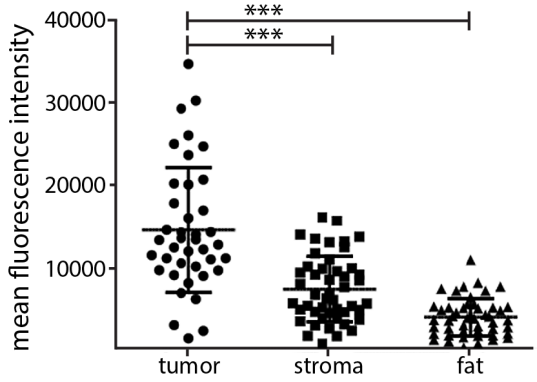


F

fluorescence scan + segmentation



G



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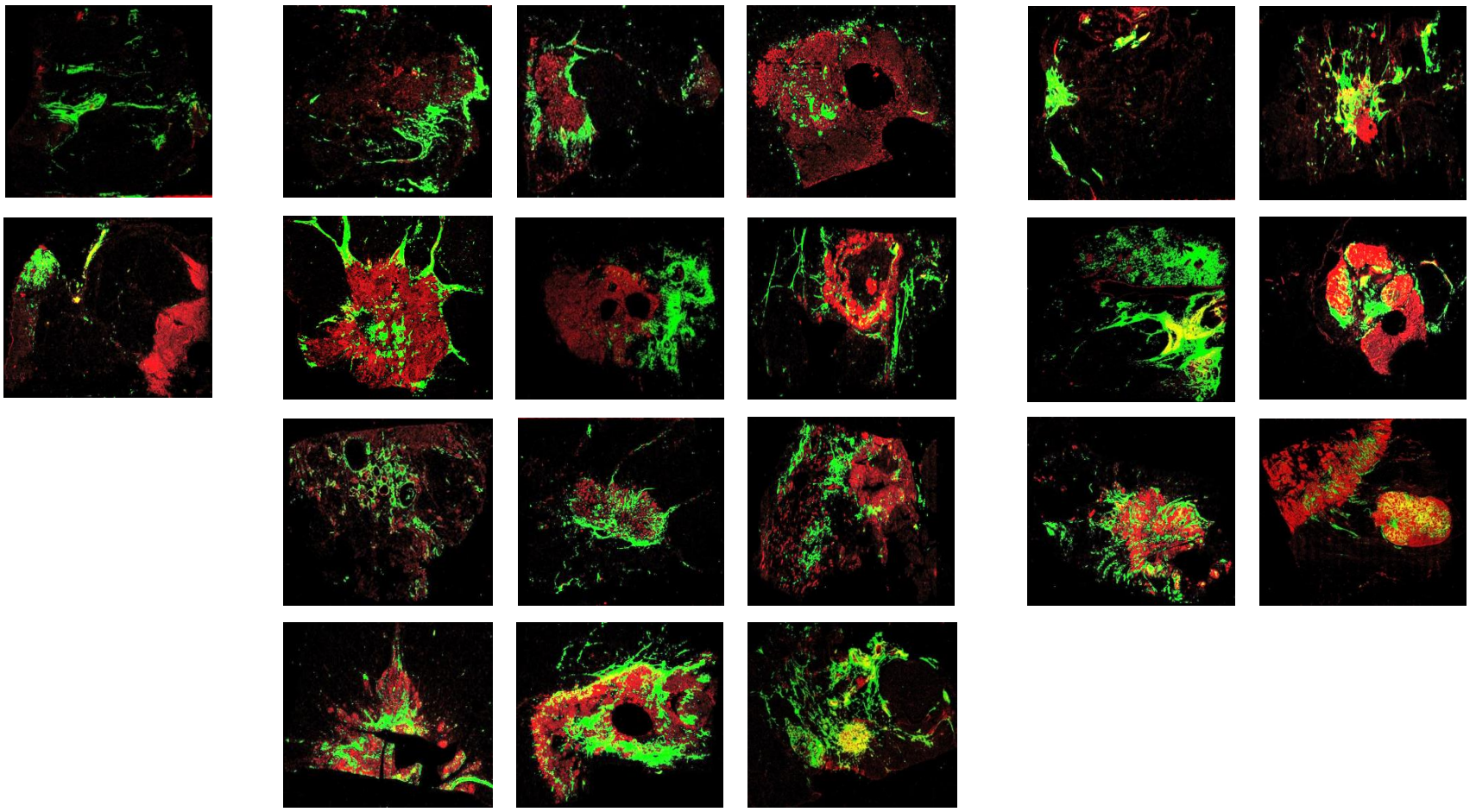
Figure 5