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Back-table fluorescence-guided imaging for circumferential resection margin evaluation in locally advanced rectal cancer patients using bevacizumab-800CW

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ABSTRACT

Rationale: Negative circumferential resection margins (CRM) are the cornerstone for curative treatment of locally advanced rectal cancer (LARC). However, in up to 18.6% of patients tumor-positive resection margins are detected upon histopathology. In this proof-of-concept study, we investigated the feasibility of optical molecular imaging as a tool to evaluate the CRM directly after surgical resection in order to improve tumor-negative CRM rates. **Methods:** LARC patients that were treated with neoadjuvant chemoradiotherapy received an intravenous bolus injection of 4.5 mg bevacizumab-800CW, a fluorescent tracer targeting vascular endothelial growth factor A (VEGFA), 2-3 days before surgery (NCT01972373). To evaluate the CRM status, back-table fluorescence-guided imaging (FGI) was performed of the fresh surgical resection specimens (N = 8). These results were correlated to histopathology. Secondly, to determine the sensitivity and specificity of bevacizumab-800CW for tumor detection, a mean fluorescence intensity (MFI) cut-off value was determined on the formalin-fixed tissue slices (N = 42; 17 patients). Local bevacizumab-800CW accumulation was evaluated by fluorescence microscopy. **Results:** Back-table FGI correctly identified a tumor-positive CRM by high fluorescence intensities in one of two patients (50%) with a tumor-positive CRM. The other patient showed low fluorescence intensities, although (sub-)millimeter tumor deposits were present <1 mm of the CRM. FGI correctly identified 5/6 tumor-negative CRMs (83%). The one patient with false-positive findings had a marginal negative CRM of only 1.4 mm. ROC analysis of fluorescence intensities of formalin-fixed tissue slices gave an optimal MFI cut-off value for tumor detection of 5,775 (sensitivity and specificity of 96.16% and 80.39% respectively). Bevacizumab-800CW enabled a clear differentiation between tumor and normal tissue up to a microscopic level, with a tumor-to-background ratio of 4.7 ± 2.5 (mean ± SD). **Conclusion:** In this proof-of-concept study, we showed the potential of back-table FGI to evaluate the CRM status in LARC patients. Optimization of this technique with adaptation of standard operating procedures could change perioperative decision-making with regard to extending resections or applying intraoperative radiation therapy in case of positive CRMs.

Key Words: optical molecular imaging; vascular endothelial growth factor a; near-infrared fluorescence; back-table fluorescence-guided imaging.

INTRODUCTION

In today's clinical practice, patients with locally advanced rectal cancer (LARC) receive long-course neoadjuvant chemoradiotherapy (nCRT) followed by surgical resection using the total mesorectal excision (TME) principles. nCRT induces tumor downsizing and downstaging, which facilitates complete resection by TME, resulting in significantly reduced local recurrence rates and increased opportunity for sphincter-sparing resections (*1*-*5*).

Obtaining negative circumferential resection margins (CRM) is key in rectal cancer therapy. The CRM has proven to be one of the most important predictors for local recurrence and to a lesser extent the development of distant metastases and survival (*6*). Restaging after nCRT occurs through a high-resolution MRI-scan for tumor staging and a CT-scan of thorax and abdomen for distant metastasis and lymph node staging (*7*-*9*). Accurate restaging can be highly challenging as differentiating between desmoplastic reaction and viable tumor tissue on preoperative imaging modalities is often difficult after nCRT.

Intraoperatively, surgeons mainly rely on visual and tactile inspection for margin assessment and differentiation between tumor and healthy tissue. This is often inaccurate, especially after nCRT, as small tumor deposits are frequently present within fibrotic parts (*10*). When in doubt, resection margins can be evaluated intraoperatively by frozen section pathologic evaluation, but this is time-consuming, costly and poses a high risk of sampling error (*11*).

Despite nCRT, TME-surgery and frozen section analysis, a tumor-positive CRM, defined as tumor presence ≤1 mm from the CRM, is detected in up to 18.6% of primary LARC surgeries upon final histopathology (*7*-*9*). Peri-operative resection margin evaluation using optical molecular imaging could improve negative CRM rates by extending resections, applying intraoperative radiation therapy (IORT) or more innovative treatment modalities like photo-immunotherapy of the wound bed (*12*). In contrast, when a margin is evaluated to be tumor-negative, extended resections could be avoided.

The aim of this proof-of-concept study was to evaluate if back-table fluorescence-guided imaging (FGI) using the near-infrared fluorescent tracer bevacizumab-800CW, could aid in evaluating the CRM status at the surgical theater. Bevacizumab-800CW targets vascular endothelial growth factor A (VEGFA),

which is overexpressed in LARC as well as many other solid tumors (*13-15*). We retrospectively analyzed back-table FGI data of fresh surgical specimens of LARC patients that were treated with nCRT. This technique may eventually allow real-time determination of CRM during surgery, which could aid intraoperative clinical decision-making with regard to extending resections or applying IORT in case of a positive CRM, to improve the outcome of LARC patients.

MATERIAL AND METHODS

Study Design and Population

Postoperative fluorescence imaging data were collected from 25 LARC patients enrolled in a clinical trial evaluating VEGFA-targeted fluorescence molecular endoscopy (Clinicaltrials.gov identifier: NCT01972373). Eligibility criteria included histologically proven LARC, with the inferior margin within 16 cm from the anal verge and treatment with long-course nCRT. To determine if back-table FGI could aid in evaluating the CRM status, patients were included if fluorescence imaging data was available from at least the anterior and posterior sides of the fresh surgical specimen. Furthermore, to evaluate local bevacizumab-800CW accumulation and determine the sensitivity and specificity of bevacizumab-800CW for tumor detection using a fluorescence cut-off value, patients with high-resolution fluorescence images available of formalin-fixed tissue slices were included. The study was performed at the University Medical Center Groningen, approved by the Institutional Review Board (METc 2013/067) and all subjects signed a written informed consent.

Surgery

After nCRT, all patients received an intended curative resection by either low anterior resection (LAR) for proximal rectum tumors or abdominal perineal resection (APR) for distal rectum tumors. Resections were extended outside the TME planes in case of tumor growth into adjacent organs. Patients only received IORT if judged necessary based on preoperative suspicion of mesorectal fascia involvement or intraoperative evaluation by the surgeons.

Histopathological Processing

Histopathological processing of surgical specimens was performed by a board-certified gastrointestinal pathologist. After gross pathology, CRMs were inked black and staple lines were removed. Specimens were anteriorly opened from proximal to distal, except for specimens with an anterior lesion, which were opened until the rectal fold. All specimens were formalin-fixated for at least 48 hours and serially sliced perpendicular to the rectum, from distal to proximal into ±0.5 cm thick tissue slices. Distal and proximal resection surfaces plus additional areas of interest (i.e. regions suspect for CRM involvement, perineural growth, vascular invasion, lymph nodes, etc.) were included for paraffin embedding. Formalin-fixed paraffin-embedded (FFPE) tissue blocks were cut in 4 μm tissue sections and hematoxylin eosin (HE) stained for routine histopathological examination. Imunohistochemistry was performed if required. A tumor-positive CRM was defined as tumor presence ≤1 mm from the inked CRM, in accordance with the Dutch national guidelines.

Bevacizumab-800CW

All patients received a 4.5mg IV bolus injection of bevacizumab-800CW (1 mg/ml) two-to-three days before surgery based on microdosing regulations (Fig. 1). Bevacizumab-800CW was produced under cGMP conditions at the University Medical Center Groningen, as described previously (*16*).

Back-table FGI

To evaluate the CRM status, back-table FGI of the fresh surgical specimen was performed in a light-tight room directly after surgery using the Explorer Air fluorescence camera (SurgVision BV, Groningen, The Netherlands, Fig. 1). Areas with high fluorescence signals were marked with a pin and subsequently inked with a different color from the CRM during pathological processing, to ensure an accurate correlation of fluorescence with histopathology.

Fluorescence Imaging of Tissue Slices and Sections

To evaluate the local tracer accumulation, fluorescence imaging of both sides of all formalin-fixed tissue slices was performed using the Explorer Vault, a standardized and light-tight fluorescence imaging system (SurgVision BV, Groningen, The Netherlands). Thereafter, two-to-three tissue slices containing tumor and/or normal rectal tissue were imaged per patient using the high resolution Odyssey CLx fluorescence imaging system (LI-COR Biosciences Inc., Lincoln, NE; Fig. 1). Additional areas of interest based on fluorescence imaging were also paraffin embedded, sliced in 4 μm tissue sections and HE stained. All FFPE tissue blocks were fluorescently scanned using the Odyssey CLx (Fig. 1). For comparison purposes, all fluorescence images were thresholded to minimum-maximum values per imaging modality for each individual patient.

To broaden our understanding of the overall penetration, distribution, and accumulation of bevacizumab-800W in rectal cancer tissue, a combination of optical tissue clearing and light-sheet fluorescence microscopy (LSFM) was performed on selected tissue slices, as previously described for preclinical tissue samples (Supplemental Material)(*17*).

Fluorescence Grid Analysis

Finally, to evaluate the sensitivity and specificity of bevacizumab-800CW for tumor detection, a fluorescence grid analysis was used based on histopathology as a gold standard to determine a fluorescence cut-off value (Supplemental Material).

Statistical Analysis

Normally distributed data is presented as mean values with standard deviation (SD) and skewed data as median values with interquartile range (IQR). A receiver operating characteristics (ROC) curve was plotted to determine the MFI cut-off value for tumor detection. P-values <0.05 were considered statistically significant. Statistical analyses were performed using Prism (version 7.0, GraphPad Software).

RESULTS

Patient Characteristics

In this retrospective proof-of-concept study, eight of 25 patients met the criteria to determine the feasibility of back-table FGI for the evaluation of the CRM status (Supplemental Fig. 1). For the second aim of this study, to evaluate local bevacizumab-800CW distribution and determine the sensitivity and specificity of bevacizumab-800CW for tumor detection on high-resolution tissue slices, 17/25 patients met the inclusion criteria (Supplemental Fig. 1). Patient characteristics are depicted in Table 1. All patients received 4.5 mg bevacizumab-800CW IV, 2-3 days prior to surgery. Ten patients underwent a LAR and seven patients an APR. There were no tracer-related (serious) adverse events in any of the patients.

Evaluation of the CRM Status

Of eight evaluated cases, two patients (25%) presented with a tumor-positive CRM on final histopathology. Back-table FGI correctly predicted the tumor-positive CRM in one of these patients due to increased fluorescence signals in the CRM (50%, Fig. 2). This patient was treated with an APR with en-bloc resection of the sacrum and a wide perineal excision because of a fistula. Intraoperatively, two frozen section analyses were performed of the lateral resection surface, both of which were benign. Interestingly, during back-table FGI of the fresh surgical specimen, high fluorescence signals were observed at the lateral resection margin (Fig. 2A). Fluorescence imaging of the correscopnding tissue slice also showed high fluorescence that seemed to reach into the CRM, while the surrounding non-tumor tissue showed low fluorescence (Fig. 2B). Final histopathology proved that this exact location was a tumor-positive CRM (Fig. 2C; orange arrows).

The second patient with a tumor-positive CRM received a LAR. Back-table FGI of the fresh surgical specimen and subsequent fluorescence imaging of the tissue slices showed low fluorescence intensities in the CRM, apart from fluorescence in two enlarged suspicious lymph nodes that proved to be tumor-positive (Supplemental Fig. 2A). However, the pathologist reported a tumor-positive CRM that was solely based on the presence of isolated microscopic vital-looking tumor deposits of (sub-)millimeter size

within a distance of 0.2-1 mm of the CRM (Supplemental Fig. 2B; orange arrow). The tumor volume was limited due to the (sub-)millimeter size of the tumor deposits, which may explain the negative imaging results.

Six out of 8 patients (75%) had a tumor-negative CRM, of which five were correctly predicted by low fluorescence intensities during back-table FGI (representative example in Supplemental Fig. 3). Subsequent fluorescence imaging of tissue slices also showed low fluorescence in relation to the CRM, although fluorescence was observed in the (intra)luminal tumor, except for the patient with a pathological complete response. IORT was applied to one of these patients due to a macroscopic suspicion of a tumor-positive CRM, while histopathology showed a tumor-negative CRM (>1 cm).

The remaining patient with a tumor-negative CRM received a low-anterior resection with en-bloc resection of the uterus, cervix, adnexa and the distal right ureter due to tumor ingrowth. Increased fluorescence was observed at the cervix during back-table FGI and subsequent fluorescence imaging of the tissue slices, potentially indicating a tumor-positive CRM. Although the fluorescence colocalized with a tumor deposit upon histopathology, the CRM was defined as tumor-negative, as the distance to the CRM was 1.4 mm.

Fluorescence Cut-off Value and Local Bevacizumab-800CW Accumulation

High-resulution fluorescence Odyssey scans of formalin-fixed tissue slices (n = 42) available from 17 patients were used to determine a semi-quantitative fluorescence cut-off value for tumor detection. A total of 5,101 grid-squares were analyzed, of which 446 were classified as tumor-positive and 4,655 as tumor-negative (Figs. 3A-3C). Significantly higher fluorescence intensities were observed in tumor areas (median MFI: 12,000) compared to surrounding non-tumor tissue (median MFI: 2,140; $P < 0.0001$; Fig. 3D), which resulted in a ratio between tumor-to-surrounding tissue of 4.7 ± 2.5 (mean ± SD). Receiver operating characteristics (ROC) curves were plotted per patient (Fig. 3E) and for all patients combined, with an area under the curve of 0.94 (std. error 0.0039; Fig. 3F). In our limited sample size, an optimal MFI

cut-off value of 5,775 was calculated using Youden's J statistics (J = 0.77), with a sensitivity and specificity of 96.19% and 80.39% respectively.

Finally, we evaluated the distribution and accumulation of bevacizumab-800CW on a microscopic level. Bevacizumab-800CW was mainly localized in the micro-environment of the tumor cells (Fig. 4), which was in line with the expected location of VEGFA (Supplemental Video 1).

DISCUSSION

Accurate perioperative evaluation of resection margins is highly important for the prognosis of LARC patients. In this explorative study, we demonstrate for the first time the feasibility of back-table FGI for identification of tumor-positive resection margins in LARC patients. Our data suggests that FGI may have the potential to guide current clinical decision-making with regard to additional targeted resections or application of IORT. Future studies using a standardized imaging protocol with larger patients samples should confirm these results.

Over the past decade, optical molecular imaging has predominantly been applied for intraoperative guidance and surgical navigation. Two clinical studies have demonstrated the feasibility and potential benefit of fluorescence-guided surgery in colorectal cancer patients (*18,19*). However, fluorescence-guided surgery is subject to several limitations related to the currently available hardware, such as variation in image-acquisition parameters and interference of ambient light. Moreover, homogenous tracer excitation is difficult especially in rectal cancer surgery, as the pelvic region is a confined surgical field often situated deep in the patient. Possibly, the development of fluorescence laparoscopes and robotic systems sensitive enough to detect micro-dosed fluorescent tracers may enable more reliable intraoperative evaluation of rectal cancer resection margins in the future.

Back-table FGI circumfents these limitations, as it makes use of a controlled, standardized and closed-field imaging environment that results in a consistent field-of-view, imaging distance and image-acquisition parameters (*20*). This enables a highly sensitive and semi-quantitative evaluation of resection margins, within a maximum of one hour after specimen excision at the OR.

The potential added value of back-table FGI was clearly demonstrated by the patient in which a tumor-positive CRM was detected at the OR, while surgical assessment and frozen section analysis were false-negative. Although intraoperative frozen section analysis is recommended for low and mid rectal tumors, it is prone to sampling error, labor-intensive and significantly prolongs anesthesia (*21,22*). By 'bringing pathology to the operating theatre' using back-table FGI, both surgeons and pathologist may be guided in correctly assessing the CRM status and evaluating the need for extended surgery or application of IORT, thereby improving personalized treatment.

In rectal cancer surgery, caution is to be taken with unnecessarily extending TME surgery to a partial or total resection of adjacent pelvic organs or applying IORT, as this can result in substantial postoperative complications and is associated with the need for reinterventions (*23*). Back-table FGI correctly predicted five out of six tumor-negative CRMs, which might have prevented unnecessary IORT application in one patient. In contrast, one close margin of 1.4 mm was identified as tumor-positive based on fluorescence. Although currently a tumor-positive margin is defined as tumor cells ≤1 mm of the CRM, this definition is under debate, as both patients with a CRM of 0.0 - 1.0 mm and 1.1 – 2.0 mm are shown to have an equally increased 2-year risk of local recurrence and distant metastases (*8,24*). Application of fluorescence may highlight the location of a (potentially) tumor-positive CRM, which allows the surgeon to perform more 'targeted' intraoperative frozen section analysis.

FGI may also support the pathologist by differentiating tumor from healthy tissue, i.e. fluorescence-guided pathology. Currently, tissue sampling is performed by gross examination of the surgical specimen and tissue slices, which can be challenging as the tissue architecture is changed by nCRT. Using the fluorescence-grid analysis, we showed a tumor-to-background ratio of 4.7 ± 2.5 on tissue slices, with a high sensitivity (96.19%) and specificity (80.39%) for tumor detection. A fluorescence cut-off value provides more objective information and may enable targeted tissue sampling, potentially saving labor, time and money. In addition, the fluorescence grid analysis can be used to evaluate tracer biodistribution and can easily be implemented on other closed-field imaging devices.

This study has several limitations. First of all, the number of patients included for CRM evaluation was relatively low, as this was a retrospective proof-of-concept analysis of a clinical trial evaluating fluorescence molecular endoscopy (NCT01972373), and imaging techniques as well as *ex vivo* imaging procedures have developed throughout the study. Secondly, a dose-escalation was not incorporated in this study design. Currently available evidence from two bevacizumab-800CW dose-escalation studies suggests that increasing the dose above microdosing levels may result in higher tracer accumulation without significantly increasing background fluorescence (*25,26*). This may further improve the detection of tumor-positive CRMs that are based on small tumor deposits, which proved to be challenging in one of our patients.

We propose a standardized imaging protocol that can be used in future research and for the development of FGI, based on our findings in the current study and our recently reported analytical workflow (Figure 5)(*26*). Fluorescence imaging is performed at fixed time-points. First, when feasible, intraoperative fluorescence-guided surgery is performed. After resection, back-table FGI of the fresh surgical specimen is performed to evaluate the resection margin status, preferably using a closed-field imaging system. In case high fluorescence is observed during intraoperative or back-table FGI, several treatment options can be considered, such as frozen section analysis, an additional or extended resection, IORT or potentially more innovative treatment modalities like photo immunotherapy (*12*). Subsequently, to correlate fluorescence with histology, fluorescence imaging is performed during histopathological processing: of the fresh surgical specimen, formalin-fixed tissue slices and paraffin-embedded tissue sections. Additionally, fluorescence microscopy can evaluate local tracer accumulation. Alltogehter, this can give more insight in tissue biodistribution, important for tracer or drug development, or to evaluate potential off- and on-target effects of fluorescence tracers. We believe such a standardized imaging protocol is widely applicable for the validation of FGI in different tumor types and with different fluorescent tracers.

CONCLUSION

Our study shows the potential of back-table FGI using the near-infrared fluorescent tracer bevacizumab-800CW targeting VEGFA for margin evaluation at the surgical theatre in patients with LARC. The technique itself proved to be safe, feasible and shows potential to guide perioperative clinical decision-making with a high sensitivity in patients with a threatened tumor-positive resection margin. A phase II study using a standardized imaging protocol is in development to confirm these results. Future studies will provide evidence if FGI will be beneficial for all patients, or will only be applied in selected cases to guide clinical decision-making.

DISCLOSURE

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KEY POINTS

Question: Can back-table fluorescence-guided imaging (FGI) be used as a tool to evaluate the circumferential resection margin status at the operating theatre, to improve tumor-positive resection margin rates in locally advanced rectal cancer patients?

Pertinent findings: In this explorative study, we demonstrate for the first time the potential of back-table FGI for identification of tumor-positive resection margins in LARC patients. In addition, we provide a data collection and data analysis design for future studies evaluating the added value of optical molecular imaging for evaluation of resection margins.

Implications for patient care: Accurate intraoperative detection of tumor-positive resection margins by back-table FGI has the potential to improve clinical decision making in locally advanced rectal cancer with regard to extending resection margins or applying intraoperative radiation therapy.

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TABLES AND FIGURES

	CRM evaluation (N = 8)		Fluorescence cut-off value (N = 17)	
	No.	%	No.	%
Sex				
Male	5	62.5%	12	70.6%
Female	3	37.5%	5	29.4%
Age (years)				
Median (range)	56 (54 - 61)		56 (31 - 76)	
Duration between nCRT - Surgery (days)				
Median (interquartile range)	87 (76-111)		87 (77-117)	
Surgery				
Low-anterior resection	5	62.5%	10	58.8%
- Including adjacent organs	1	-	2	-
Abdominoperineal resection	3	37.5%	7	41.2%
- Including adjacent organs	3	-	5	-
Intraoperative radiation therapy				
Not standby	3	37.5%	-	-
Standby	4	50%		
Applied	1	12.5%		
Histopathological staging				
pT0 N0 M0 (pCR)	1	12.5%	1	5.9%
pT2 N0 M0	2	25.0%	2	11.8%
pT3 N0 M0	1	12.5%	4	23.5%
pT3 N1 M0	0	-	3	17.6%
pT3 N2 M0	2	25.0%	5	29.4%
pT4 N0 M0	2	25.0%	2	11.8%
Circumferential resection margin (CRM)				
≤ 1 mm (tumor-positive)	2	25.0%	3	17.6%
1-2 mm	1	12.5%	2	11.8%
> 2 mm	4	50.0%	11	64.7%
pCR	1	12.5%	1	5.9%
Distal resection margin				
≤ 1 mm (tumor-positive)	0	-	0	-
1-2 mm	0	-	1	5.9%
> 2 mm	7	87.5%	15	88.2%
pCR	1	12.5%	1	5.9%

TABLE 1

Patient and tumor characteristics. nCRT = neoadjuvant chemoradiotherapy; pCR = pathological complete response; TNM = Tumor, Node, Metastasis.

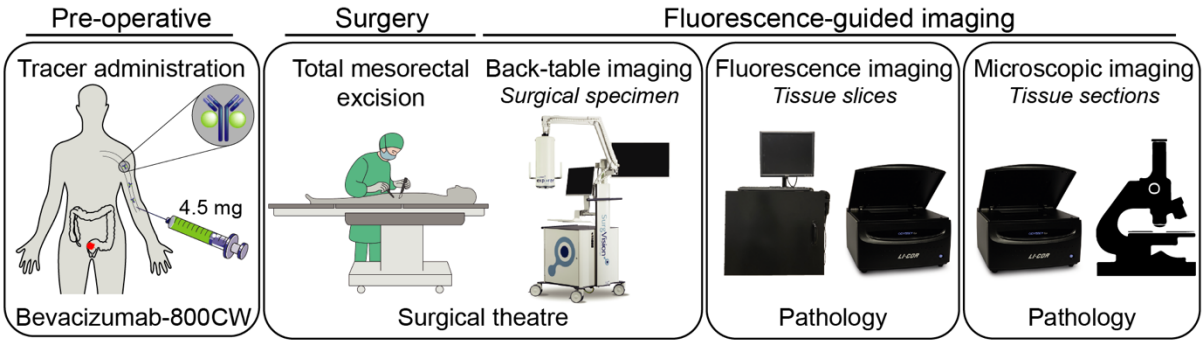


FIGURE 1

Schematic overview of study design. Bevacizumab-800CW (4.5 mg) was administered intravenously 2-3 days prior to surgery. Fluorescence-guided imaging was performed at every step during pathological processing: back-table of the fresh surgical specimen, of the tissue slices and tissue sections. Results were correlated to histopathology.

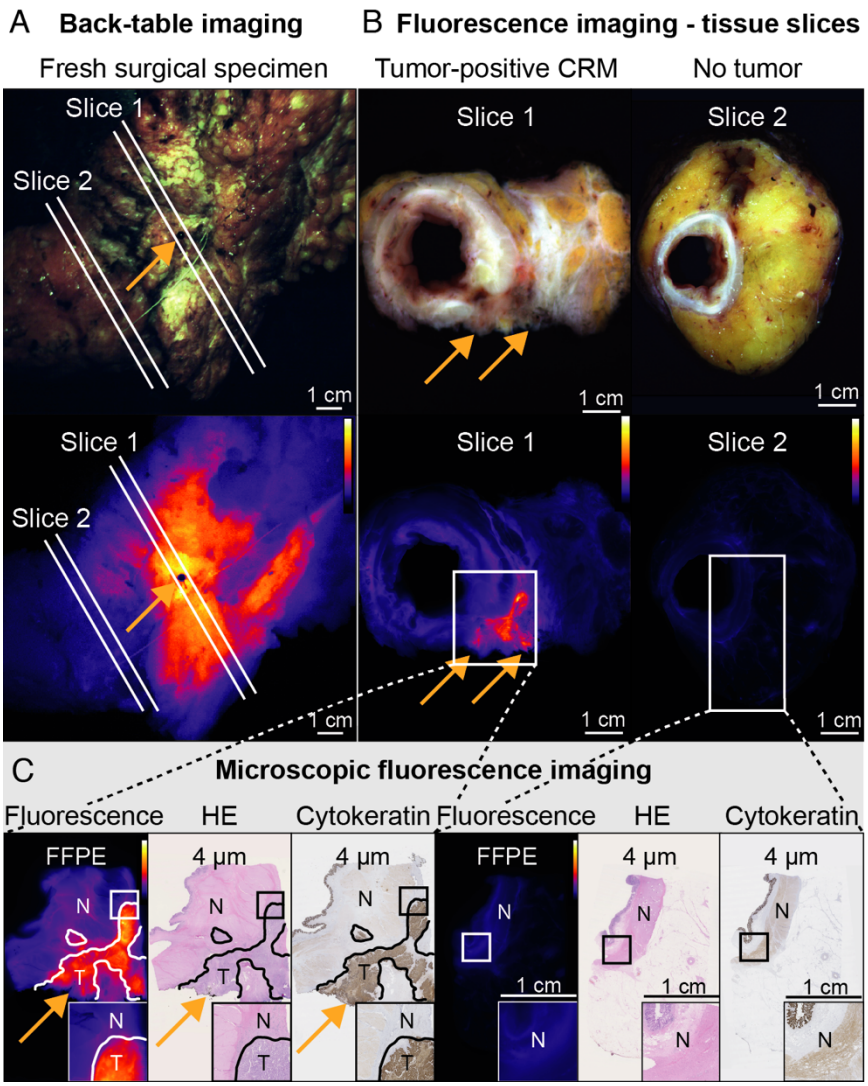


FIGURE 2

Back-table FGI of a patient with a tumor-positive CRM, with a black pin at the location showing increased fluorescence, to enable an accurate correlation with histology (A, orange arrow). Fluorescence imaging of two corresponding tissue slices (B) and further microscopic fluorescence imaging and histological correlation (C), with orange arrows indicating the location of the tumor-positive CRM. High fluorescence is observed at the CRM of tissue slice 1 containing the tumor-positive CRM, whereas low fluorescence was observed in the non-tumor tissue slice 2, corresponding to the microscopy results. FFPE = formalin-fixed paraffing embedded; HE = hematoxylin eosin; N = non-tumor; T = tumor.

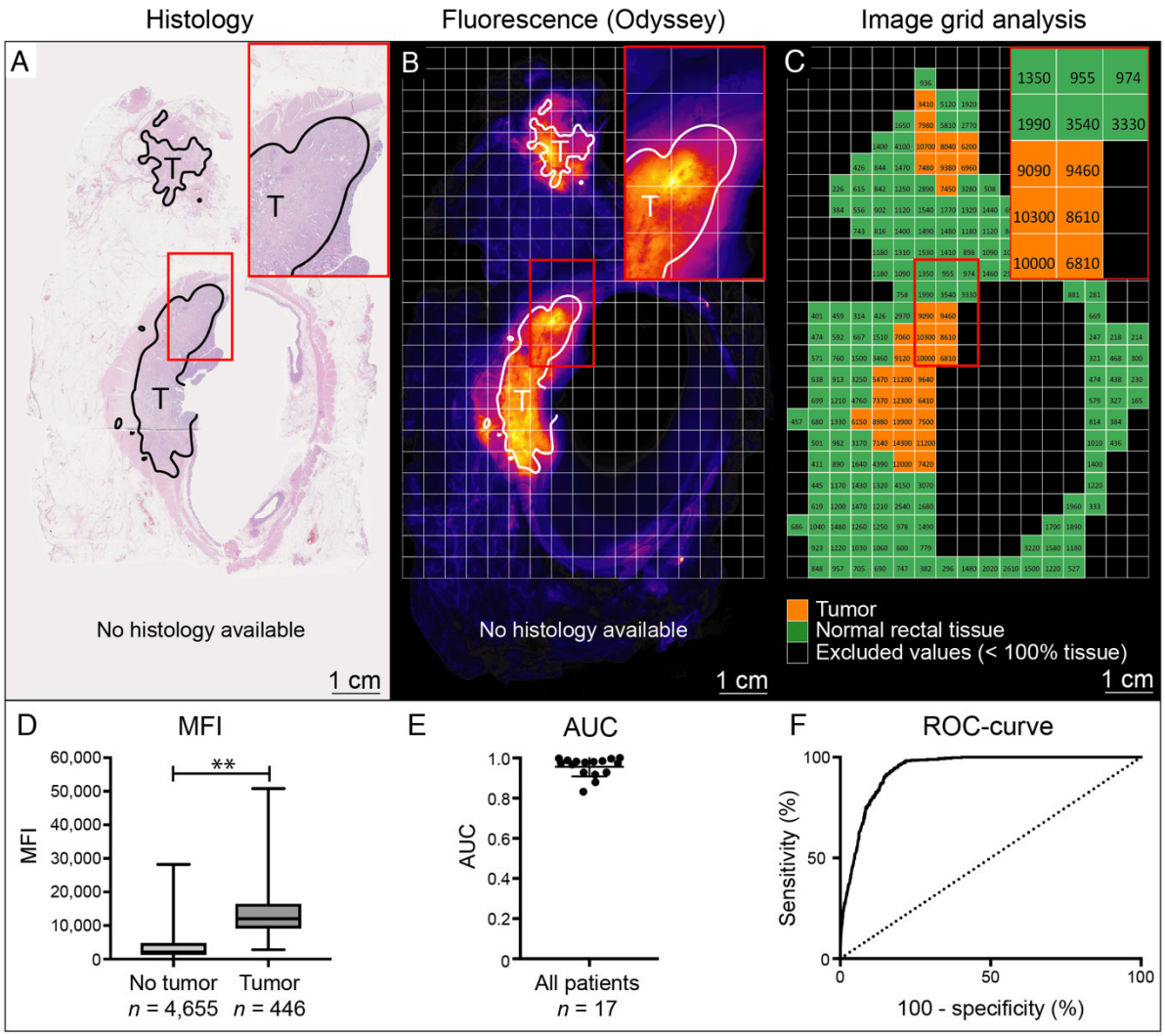


FIGURE 3

Fluorescence grid analysis. All tumor tissue was delineated on a hematoxylin eosin (HE)-staining (A) and subsequently overlaid on the high-resolution fluorescence images of tissue slices (B). Each 3-by-3 mm square was selected as tumor-positive (C, orange) or tumor-negative (C, green). Median fluorescence intensities (MFI) were calculated per square, with significantly higher fluorescence in tumor tissue compared to surrounding non-tumor tissue (12,000 versus 2,140; $P < 0.001$; D). Individual areas under the curve were determined per patient (E) and a receiver operating characteristic (ROC)-curve was plotted, showing an area under the curve (AUC) of 0.94 (F).

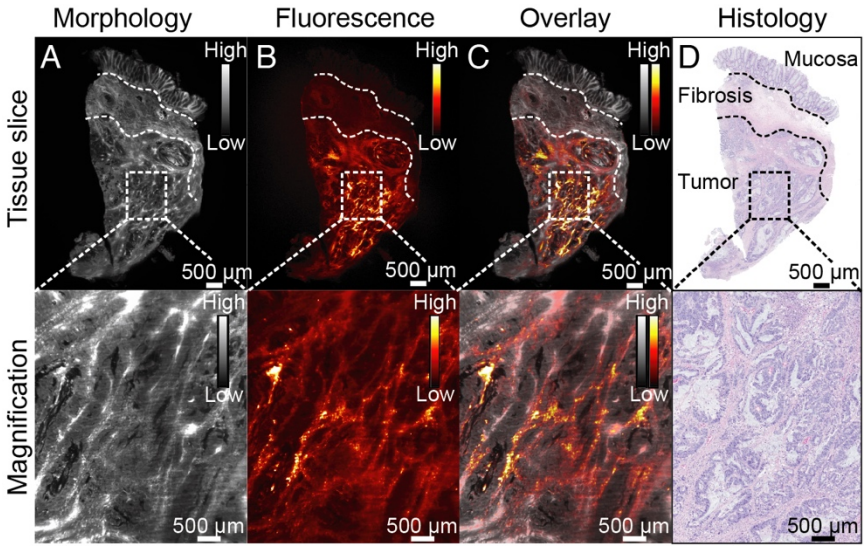


FIGURE 4

Representative example of light-sheet fluorescence microscopy to evaluate the local tracer accumulation in a rectal cancer tissue slice, showing the tissue morphology based on autofluorescence (A), the fluorescence (B), an morphology and fluorescence overlay (C) and the corresponding histology (D). Magnified images are depicted in the bottom row. Increased bevacizumab-800CW binding can be seen in the micro-environment of the tumor cells compared to surrounding normal mucosa and fibrosis.

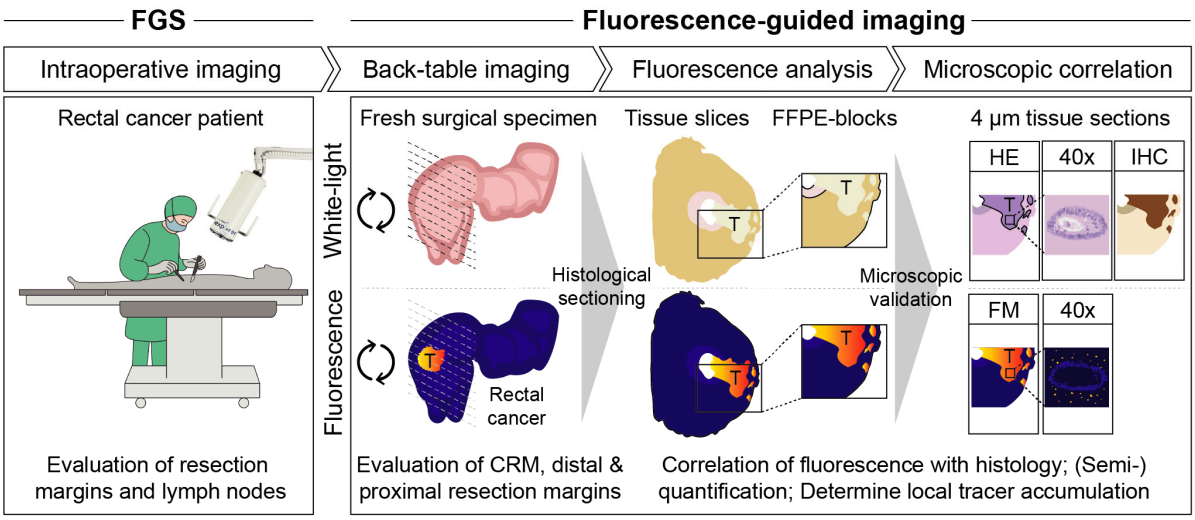


FIGURE 5

Proposed data collection and analysis design for resection margin evaluation, including intraoperative imaging to evaluate resection margins and identify potential lymph nodes and/or peritoneal metastasis, back-table imaging of the fresh surgical specimen to evaluate resection margins, and further fluorescence analysis of tissue slices, formalin-fixed paraffin-embedded (FFPE) tissue blocks and tissue sections for cross-reference and correlation of fluorescence with histology. T = tumor; HE = hematoxylin eosin; IHC = immunohistochemistry; FM = fluorescence microscopy.

MATERIAL AND METHODS

Fluorescence Grid Analysis

Tumor locations were delineated on 4 µm HE stained tissue sections by a board-certified gastrointestinal pathologist, who was blinded for fluorescence imaging results. The histological delineation was merged with the high-resolution fluorescence images of the formalin-fixed tissue slices (Odyssey CLx), to enable a direct correlation of fluorescence with histology. Subsequently, a fluorescence grid analysis was performed, which was adapted from Gao et al (*1*). A 3 x 3 mm grid was drawn on the merged image using ImageStudio software (version 5.0, LI-COR Biosciences Inc., Lincoln, NE, USA), dividing it into identical 9 mm^2 squares. Each square was classified as tumor-negative or tumor-positive if more than 20% of the square consisted of tumor-tissue, based on the histological delineation. ImageStudio software automatically calculated mean fluorescence intensities (MFI) for each square. A receiver operating characteristics (ROC) curve was determined per patient and for all patients combined, to determine an optimal cut-off value for tumor detection based on optimal sensitivity and specificity as determined by Youden's J statistics.

Three-Dimensional Tissue Analysis by Light-Sheet Fluorescence Microscopy.

LSFM enables a multicolor 3D analysis of optical transparent whole-mount tissue specimen at cellular resolution. Tissue slices were formalin fixated, dehydrated, and incubated in an organic clearing solution (one-part benzylalcohol & two-parts benzylbenzoate, incubation condition: 24-48 hours, 4°C, dark) to obtain high optical tissue transparency. Subsequently, the fluorescence signal intensity of bevacizumab-800CW as well as the tissue autofluorescence (providing detailed morphological tissue information) were measured within the cleared rectal cancer tissue using a commercially available light-sheet microscope (UltraMicroscope II, LaVision Biotec GmbH, Bielefeld, Germany). The obtained fluorescence imaging results were visualized as single and co-registered data sets, combining information of drug penetration/accumulation with morphological tissue context. In addition, the performed virtual 3D tissue analysis was also correlated to conventional histology.

Fluorescence imaging parameters

Throughout the study, the same imaging parameters were used for data acquisition per imaging device. In case of saturation of fluorescence images, the fluorescence exposure time or gain was decreased to ensure adequate data collection.

REFERENCE

1. Gao RW, Teraphongphom NT, van den Berg NS, et al. Determination of tumor margins with surgical specimen mapping using near-infrared fluorescence. Cancer Research. 2018;78:5144-5154.

TABLES AND FIGURES

	Tumor-positive CRM	Tumor-negative CRM	Total
High fluorescence	1	1*	**2**
Low fluorescence	1**	5	**6**
Total	**2**	**6**	**8**

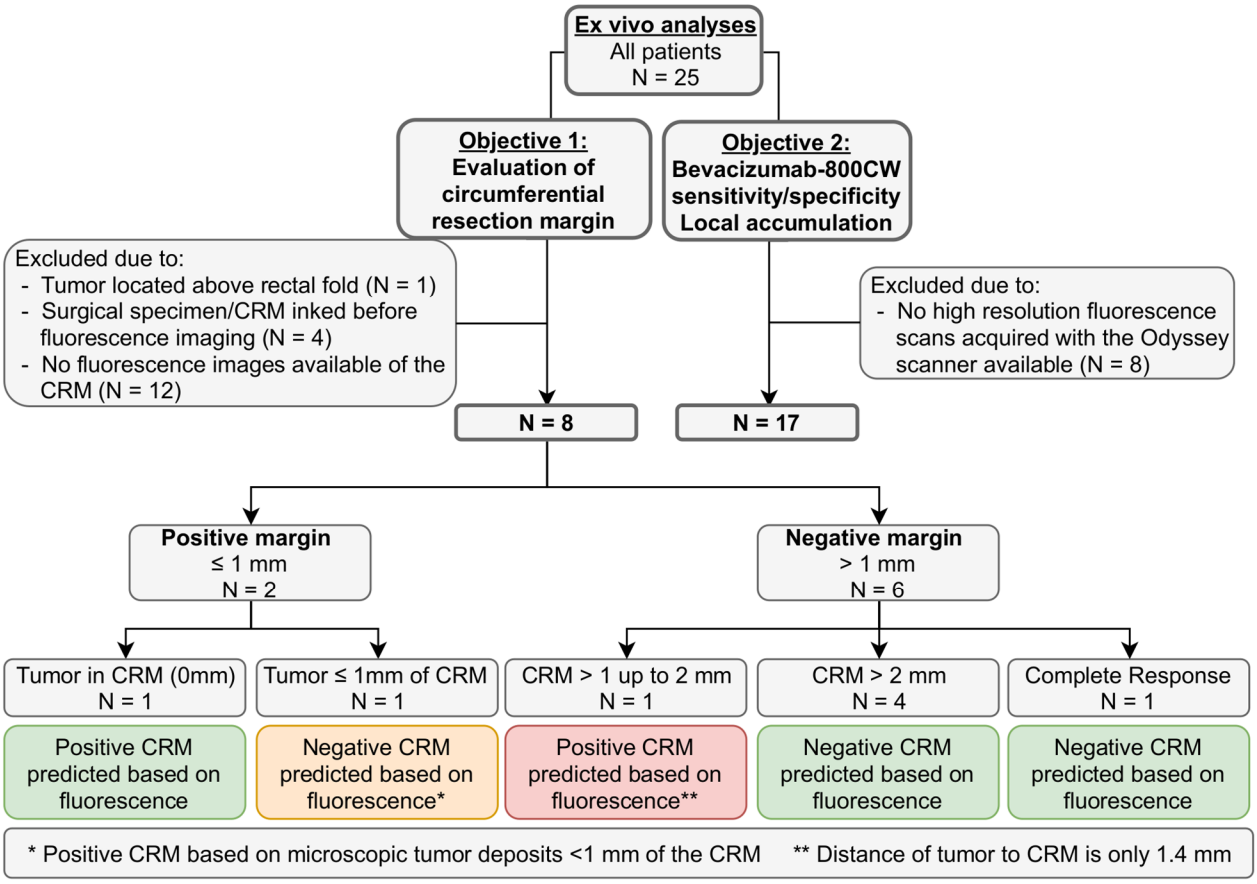
SUPPLEMENTAL TABLE 1

Contingency table. A qualitative evaluation of fluorescence intensities was performed on the fresh surgical specimens. Low fluorescence was assessed as a homogenous background fluorescence; localized increased fluorescence was assessed as high fluorescence. *Distance to the circumferential resection margin (CRM) of 1.4 mm. **Tumor-positive CRM based on isolated microscopic tumor deposits ≤ 1 mm of the CRM.

Attachment

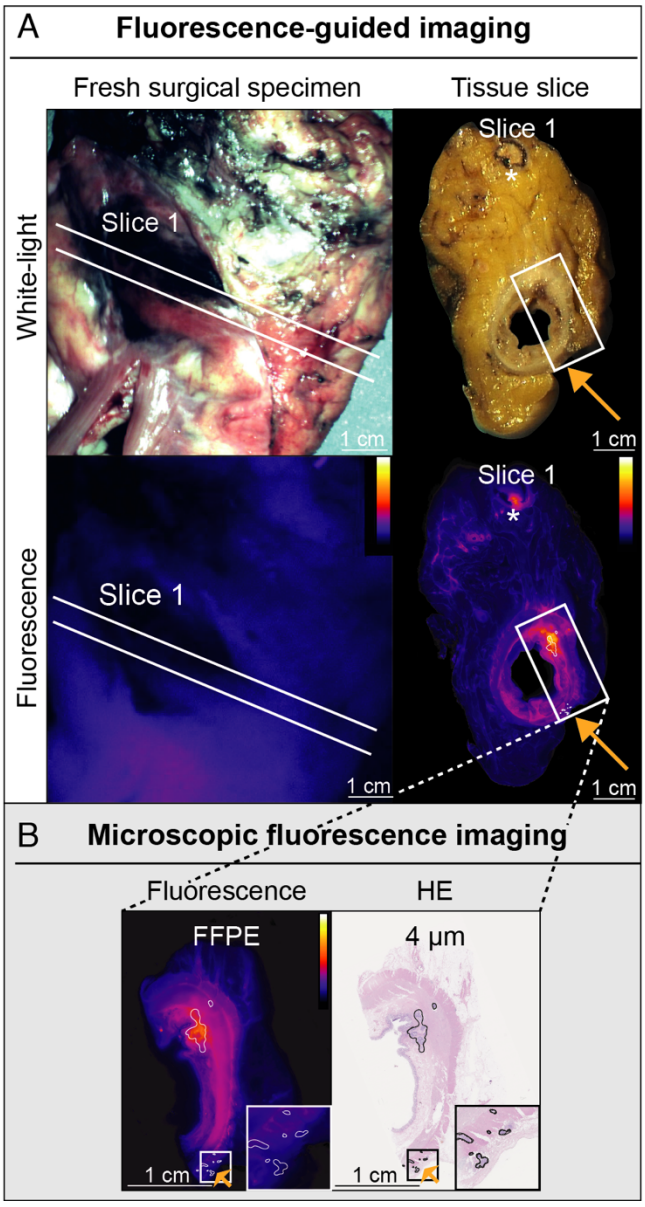
SUPPLEMENTAL VIDEO 1

Three-dimensional light-sheet microscopy of bevacizumab-800CW fluorescence localized in tumor environment of a tissue slice containing tumor. Tissue slice histology is also depicted in Figure 4. The left tissue slice depicts the tissue morphology based on autofluorescence; the right tissue slice depicts the near-infrared fluorescence image.



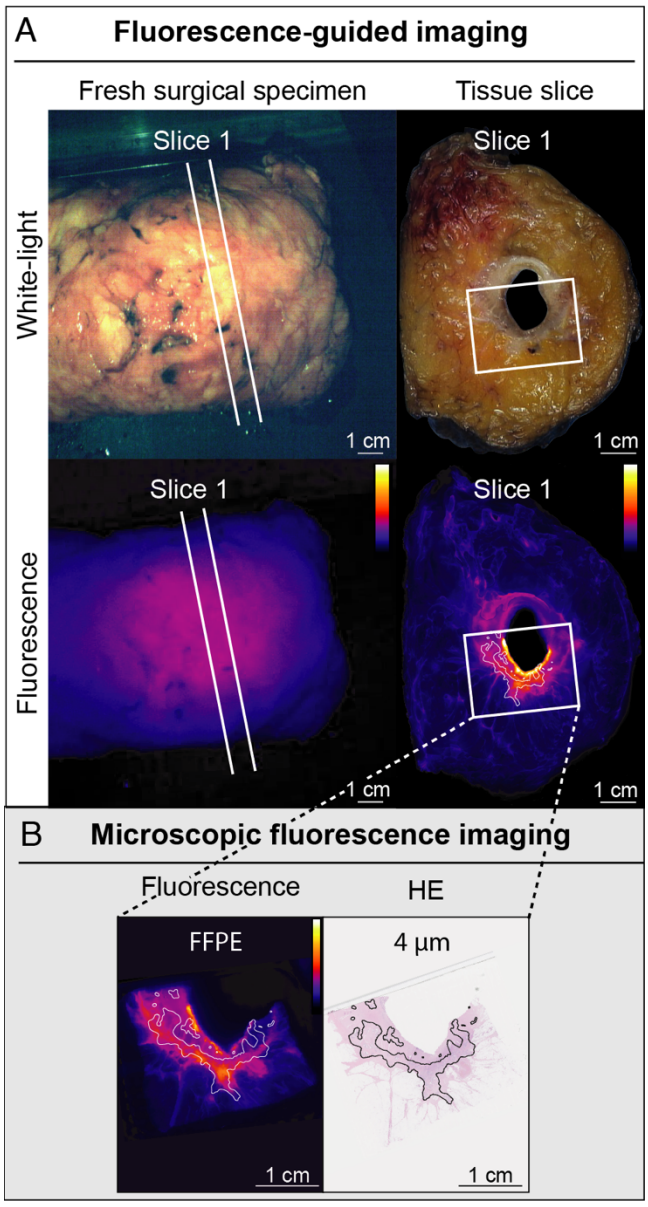
SUPPLEMENTAL FIGURE 1

Flow diagram of the study. From 25 patients, eight patients were included in this explorative analysis for the use of back-table fluorescence-guided imaging to evaluate the circumferential resection margins (CRM) status on the fresh surgical specimens. Out of two tumor-positive margins, one was identified correctly using back-table, whereas the other was considered tumor-negative, despite the presence of (sub)millimeter tumor deposits <1 mm of the CRM. Five out of six tumor-negative CRMs were identified correctly. The remaining CRM was identified to be tumor-positive based, although the CRM was marginally negative CRM with 1.4 mm. Seventeen patients were included to determine the sensitivity and specificity of bevacizumab-800CW and evaluate local tracer accumulation, including the eight patients included for CRM evaluation.



SUPPLEMENTAL FIGURE 2

Tumor-positive CRM with low fluorescence intensities during back-table FGI of the fresh surgical specimen and fluorescence imaging of a subsequent tissue slice (A). The orange arrows indicate the location of the tumor-positive CRM that was based on isolated microscopic tumor deposits as determined on final histopathology (B). The asterix (*) indicates a tumor-positive lymph node. FFPE = formalin-fixed paraffin embedded; HE = hematoxylin eosin.



SUPPLEMENTAL FIGURE 3

Tumor-negative CRM with low fluorescence during back-table FGI of the fresh surgical specimen and a corresponding tissue slice, showing fluorescence at the location of the luminal tumor, though low fluorescence near the CRM (A), which was confirmed during microscopic fluorescence imaging and correlated to histology (B). FFPE = formalin-fixed paraffin embedded; HE = hematoxylin eosin.