‘RED-FLAG’ IMAGING TECHNIQUE: IDENTIFICATION OF COLORECTAL ADENOMAS WITH WIDE-FIELD NEAR-INFRARED FLUORESCENCE ENDOSCOPY

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ABSTRACT

**Background** Miss rates in colorectal adenoma detection are unacceptably high, especially in high-risk populations, like patients with Lynch syndrome. In a clinical proof-of-principal study, we investigate near-infrared fluorescence molecular endoscopy (NIR-FME) for adenoma detection. We target the vascular endothelial growth factor A (VEGFA), which is highly upregulated in colorectal adenomas. We aimed to identify the optimal systemic dose of a fluorescently labeled monoclonal antibody against VEGFA (bevacizumab-800CW) for adenoma detection.

**Methods** Patients with familial adenomatous polyposis (FAP) (n = 17), received an intravenous injection with 4.5, 10 or 25 mg of bevacizumab-800CW, targeting VEGFA. Three days later, they received NIR-FME, with concurrent fluorescence and high definition (HD) white-light imaging. Validation of NIR-FME findings was based on ex vivo Multi-Diameter Single Fiber Reflectance and Single Fiber Fluorescence (MDSFR/SFF) spectroscopy of freshly excised specimen. Additional NIR fluorescence flatbed scanning, fluorescence microscopy and VEGFA immunohistochemistry (IHC) were performed to visualize tracer distribution at a microscopic level, enabling correlation with histopathology and protein expression.

**Results** VEGFA-targeted NIR-FME detected colorectal adenomas in all tracer cohorts. Best results were achieved in the 25 mg cohort, which even detected small adenomas (<3 mm). Spectroscopy analyses demonstrated the highest adenoma-to-normal ratio of 1.84 for the 25 mg cohort, with a calculated median tracer concentration in adenomas of 6.43 nmol/mL. Ex vivo signal analyses demonstrated NIR fluorescence within the dysplastic areas of the adenomas.

**Conclusion** NIR-FME is clinically feasible as a real-time, red-flag technique for detecting colorectal adenomas, with 25 mg bevacizumab-800CW as best-performing tracer dose. ClinicalTrials.gov ID: NCT02113202.
INTRODUCTION

Colorectal cancer (CRC) is the second most common cancer worldwide, accounting for 8% of all cancer related deaths. Of all CRCs, approximately 60% develops via the well-known adenoma-carcinoma sequence, one-third via the alternative serrated pathway and a small portion from Lynch syndrome (LS). White-light endoscopy is considered the gold standard for detection and removal of colorectal lesions, to prevent CRC development. However, detection of small adenomas (<5 mm) and sessile serrated adenomas / polyps (SSA/P) is difficult since conventional endoscopy relies upon aspecific morphologic tissue signatures and thus on the experience of the endoscopist. As a result, the reported adenoma detection miss rate for the general population is 27%, relatively high. For patients with LS, adenoma miss rates are even up to 55%. In this high-risk population small adenomas are more common, and these often already contain high-grade dysplasia (HGD) since the adenoma-carcinoma sequence is known to be accelerated. Therefore, missed lesions can rapidly progress to cancer which results in an unacceptable cumulative cancer risk of up to 35% at the age of 60, despite intensive screening programs. This underscores the necessity of improving endoscopic detection strategies.

One way to improve endoscopic lesion identification is the incorporation of wide-field fluorescence molecular endoscopy (FME). FME visualizes lesions based on their biological properties rather than their morphology; it uses exogenous fluorescent tracers that bind to specific proteins, thereby fluorescently ‘highlighting’ the tissue of interest as a ‘red-flag’ for the endoscopist. A recently published study showed a higher adenoma detection rate with FME following an IV injected anti-cMET tracer using an old white-light fiber endoscope for both fluorescence and white-light imaging, which hampers clinical translation. Moreover, FME in the visible spectrum limits the sensitivity and contrast available to the fluorescence method. Separation of weak fluorescence signals in the presence of strong white-light illumination requires several orders of magnitude spectral separation through filters, which also reduces significantly the transmission of fluorescence signals. Moreover, the visible light exhibits strong autofluorescence, reducing contrast.

Therefore, we labelled the monoclonal antibody bevacizumab with a near-infrared (NIR) fluorescent dye, IRDye 800CW, and used a NIR-FME platform that enables concurrent fluorescence and high definition (HD) white-light imaging. Bevacizumab-800CW binds vascular endothelial growth factor A (VEGFA), which is present in all stages of colorectal neoplasms, including low grade dysplastic (LGD) adenomas and even up to 90% expression in difficult to detect but important sessile serrated polyps/adenomas. We evaluated VEGFA-targeted NIR-FME for adenoma detection in a dose-escalation study, performed in patients with famil-
inal adenomatous polyposis (FAP) who have a high probability of occurrence of colorectal adenomas. We choose FAP patients due to the abundance of colorectal adenomas in this condition, to enable adequate evaluation of the different dose steps. However, we did not intend to demonstrate a potential clinical application for FAP patients. To validate our in vivo NIR-FME findings, we quantified the fluorescence of excised colorectal tissue by correcting for the influence of tissue optical properties using Multi-Diameter Single Fiber Reflectance and Single Fiber Fluorescence (MDSFR/SFF) spectroscopy.

**MATERIAL AND METHODS**

**Study population and design**

Patients with FAP that were 18 years of age or older, and scheduled for surveillance endoscopy at the University Medical Center Groningen (UMCG), were invited to participate in the study. Trial enrolment required FAP to be genetically proven or clinically diagnosed by >100 colorectal adenomatous polyps at earlier endoscopy. Patients with a MUTYH mutation or who had a proctocolectomy were excluded. The study protocol was approved by the Medical Ethics Committee of the UMCG. All patients gave their written informed consent for participation in the study before inclusion. The study was registered with ClinicalTrials.gov (NCT02113202).

This non-randomized, non-blinded, single center proof-of-principle study consisted of three tracer-dose cohorts: 4.5, 10 and 25 mg of bevacizumab-800CW (Figure 1A).\(^{10}\) These tracer dosages are low compared to the therapeutic dose of bevacizumab (5-10 mg/kg).\(^{11}\) Three days after IV tracer injection, patients underwent surveillance endoscopy with a HD endoscope followed by NIR-FME to visualize fluorescent signals. Afterwards, we validated the observed fluorescence by ex vivo signal quantification on fresh resected tissue with MDSFR/SFF spectroscopy. Additionally, to specify tracer distribution and enable correlation to histopathology, we performed NIR-fluorescence flatbed scanning, fluorescence microscopy and VEGFA immunohistochemistry (IHC) on formalin-fixed, paraffin-embedded (FFPE) tissue (Figure 1B).

In the 4.5 mg cohort, six FAP patients with adenomas were included. The interim analysis incorporated in our study protocol, allowed for a tracer dose escalation if the 4.5 mg tracer dose appeared suboptimal. As a result, three patients were included in the subsequent dose cohorts (10 and 25 mg); the best-performing tracer-dose cohort (25 mg) was expanded to six FAP patients in total. All observed AEs were noted until 72 hours after intravenous tracer injection. This period was chosen as bevacizumab-800CW had shown a good safety profile in previous studies in patients with primary breast cancer and peritoneal carcinomatosis of colorectal origin.\(^{12}\)
Figure 1. Schematic overview of the study time-line and set-up. (A) Schematic overview of the in vivo NIR-FME procedure, with intravenous administration of the fluorescent tracer bevacizumab-800CW 3 days prior to procedure. (B) Overview of the ex vivo fluorescent signal analyses: 1) quantitative signal analyses with MDSFR/SFF spectroscopy performed immediately after the procedure on the fresh resected tissue, and 2) the qualitative tracer analysis, e.g. evaluation of tracer tissue distribution performed on FFPE tissue. NIR-FME, near-infrared molecular fluorescence endoscopy; IV, intravenous; CR, colorectal; MDSFR/SFF spectroscopy, multi-diameter single fibre reflection and single fibre fluorescence spectroscopy; FFPE, formalin-fixed and paraffin-embedded; HE, haematoxylin and eosin; IHC, immunohistochemistry; VEGFA, vascular endothelial growth factor A.

NIR-FME Procedure

Endoscopy was performed after standard bowel preparation, optionally under conscious sedation with midazolam and fentanyl. All procedures were performed with a routine clinical HD video endoscope, which is standard of care during surveillance endoscopies (CF-H180AL/I or GIF-H180; EVIS EXERA II; Olympus Corporation, Tokyo, Japan). White light was provided by a standard xenon light source (CLV-180 Evix Exera II; Olympus Corporation), in which a short pass filter was installed (<750 nm, E700SP-2P; Chroma, Bellows Falls, VT, USA). When the caecum or ileorectal anastomosis was reached, the NIR-FME probe was introduced in the working channel of the video endoscope (supplementary figure 1A). During withdrawal, adenomas were concurrently visualized with both the video endoscope and the NIR-FME probe. The NIR-FME images (color, fluorescence and overlay) were displayed on a separate monitor (Figure 2). Subsequently, all large adenomas (>5 mm; standard clinical care) and a maximum of six small adenomas (<3 mm) were excised. Additionally, four biopsies of normal appearing colorectal mucosa were taken for research purposes only. See online supplementary materials and methods for detailed information on tracer production and the technical background of the NIR-FME system.
Fluorescent adenomatous polyp
Emitted signal from targeted polyp
Exciting NIR signal from FME probe

Figure 2. Wide-field molecular guided near-infrared fluorescence endoscopy. (A) Endoscopic NIR imaging, based on a clinical HD video endoscope combined with a NIR fibre bundle, enabling fluorescence-based identification of lesions. (B) Demonstration of the two monitors used, visualizing both HD white-light for optimal morphology and fluorescent images, including an overlay projection. NIR, near-infrared; HD, high definition; FME, molecular fluorescence endoscopy.

MDSFR/SFF Spectroscopy
The freshly resected adenomas and normal mucosa biopsies were placed on ice with the mucosal side upwards. Directly after the endoscopy the MDSFR/SFF spectroscopy probe was placed on top of the fresh tissue for quantitative measurements of NIR fluorescence. This device gains two reflectance spectra via two different optical fibers and subsequently one raw fluorescence spectrum (supplementary figure 1B and 2). From the reflectance spectra, the scattering and absorption coefficients were determined, which were used to determine the intrinsic fluorescence. The intrinsic fluorescence was afterwards used to calculate the actual bevacizumab-800CW concentration present in the fresh resected tissue. Subsequently, the majority of resected adenomas and normal tissue biopsies were formalin-fixed and paraffin-embedded (FFPE), while some were snap-frozen in liquid nitrogen and stored at -80°C. In one patient (25 mg cohort) no quantitative measurements could be collected due to a technical malfunction of the MDSFR/SFF spectroscopy device. See online supplementary materials and methods for more detailed information on the MDSFR/SFF spectroscopy device, spectral fitting and determination of the local bevacizumab-800CW concentration.

Histological Fluorescence Mapping
Per FFPE tissue block, one 10 µm section and three 4 µm sections were sliced, mounted on silane-coated slides and dried overnight at 37°C. The 10 µm FFPE tissue sections were deparaffinized (10 min xylene) and imaged with the NIR fluorescence flatbed. Afterwards, the 10 µm tissue sections and the subsequent 4 µm tissue sections were stained with hematoxylin and eosin (HE). The HE slides were digitalized by the Nanozoomer 2.0-HT slide scanner (Hamamatsu) and viewed with use of NanoZoomer Digital Pathology (NDP) viewer software (Hamamatsu). Blinded for the fluorescence signals and under supervision of an experienced gastrointestinal pathologist (AK), different tissue areas were selected: low-grade dysplasia
(LGD) areas within the adenoma, normal adjacent tissue within the adenoma section and normal colon crypts within the normal tissue biopsies. Afterwards, these areas were superimposed on the NIR fluorescence Odyssey images. Ex vivo analyses of the snap frozen tissue was shown to be unreliable, as bevacizumab-800CW signals diminished during thawing of the samples.

**Fluorescence Microscopy**

For fluorescence microscopy, 4 µm FFPE tissue sections were deparaffinized, rehydrated and stained with Hoechst to visualize nuclei (33258; Invitrogen, Thermo Fisher Scientific). Fluorescence microscopy was performed using an inverted wide-field microscope (63-100x magnification, immersion oil; DMI6000B, Leica Biosystems GmbH, Nussloch, Germany), with a LED light source that is able to excite up to 900 nm (X-Cite 200DC; Excelitas Technologies, Waltham, MA, USA), a monochrome camera also sensitive in the NIR range (1.4M Pixel CCD, DFC365FX; Leica Biosystems GmbH) and an adapted filter set (two band-pass filters 850-90m-2p and a long-pass emission filter HQ800795LP; Chroma Technology). All tissue slides were assessed using the same settings to enable visual comparison. Following acquisition, the images were processed with LAS-AF2 software (Leica Microsystems).

**Immunohistochemical Analysis of VEGFA Expression**

We previously demonstrated the relevance of VEGFA as a target for colorectal neoplasia. To determine if the prior VEGFA results hold true for our current patient population, we immuno-stained all FFPE colorectal tissue collected during this study (polyclonal rabbit anti-human VEGFA, RB9031 1:300; Thermo Fisher Scientific, Waltham, MA, USA). To ascertain specific binding of the anti-VEGFA antibody, a positive tissue control and a negative IgG control were included. Dysplastic crypts, normal crypts within the adenoma section and normal mucosa derived from the biopsies were scored separately for their staining intensity (0-3 scale) and the percentage of cells stained. This visual scoring was performed by two separate observers (EH and JJJT). Subsequently, H-scores were generated (continuous scale: 0-300) by combining the evaluated intensity and the corresponding percentage of cells stained. See online supplementary materials and methods for detailed description of the IHC methods and H-score formula.

**Statistical analysis**

For statistical analysis of the MDSFR/SFF spectroscopy and flatbed scanning results IBM SPSS 22.0 (IBM Corporation, Armonk, NY, USA) and GraphPad Prism 5.0 (GraphPad Software Inc, La Jolla, CA, USA) were used. A two-tailed t test, Mann-Whitney U test or
Kruskal-Wallis test was used, according to sample-size and distribution. P values <0.05 were considered statistically significant. All authors had access to the study data and reviewed and approved the final manuscript.

RESULTS

Near-infrared Molecular Fluorescence Endoscopy (NIR-FME)
In total, 17 patients participated in the study (table 1). Eight patients received 4.5 mg, three patients 10 mg and another six patients 25 mg bevacizumab-800CW intravenously. No tracer-related adverse events were observed. Two patients in the 4.5 mg cohort did not have any lesions at endoscopy and were excluded from the further analyses.

Table 1.

<table>
<thead>
<tr>
<th>Patient and adenoma characteristics</th>
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<tbody>
<tr>
<td><strong>Number of patients, n (%)</strong></td>
<td></td>
</tr>
<tr>
<td>Complete colon in situ</td>
<td>17</td>
</tr>
<tr>
<td>Ileorectal anastomosis</td>
<td>5  (29.4%)</td>
</tr>
<tr>
<td></td>
<td>12  (70.6%)</td>
</tr>
<tr>
<td><strong>Sex, n (%)</strong></td>
<td></td>
</tr>
<tr>
<td>Male</td>
<td>5  (29.4%)</td>
</tr>
<tr>
<td>Female</td>
<td>12  (70.6%)</td>
</tr>
<tr>
<td><strong>Age, in years</strong></td>
<td></td>
</tr>
<tr>
<td>Median (range)</td>
<td>42  (20-65)</td>
</tr>
<tr>
<td><strong>Total number of observed adenomas per subject, n (%)</strong></td>
<td></td>
</tr>
<tr>
<td>0</td>
<td>2  (11.8%)</td>
</tr>
<tr>
<td>1-5</td>
<td>4  (23.5%)</td>
</tr>
<tr>
<td>6-20</td>
<td>10  (58.8%)</td>
</tr>
<tr>
<td>&gt;20</td>
<td>1  (5.9%)</td>
</tr>
<tr>
<td><strong>Histology of resected polyps, n (%)</strong></td>
<td></td>
</tr>
<tr>
<td>LGD adenomatous polyp</td>
<td>50  (98%)</td>
</tr>
<tr>
<td>HGD adenomatous polyp</td>
<td>1  (2%)</td>
</tr>
</tbody>
</table>

* The two subjects without any polyps at endoscopy were left out of the ex vivo fluorescent signal analyses.

For all three tracer-dose cohorts, VEGFA-targeted NIR-FME fluorescently visualized all adenomas that were identified during HD inspection (sensitivity 100%) (Figure 3A). All larger adenomas (≥5 mm) showed sufficient fluorescent contrast for in vivo detection at video rate (10 frames per second [FPS]). Small adenomas (<3 mm) showed varied fluorescence in the
4.5 mg bevacizumab-800CW dose cohort. In some cases, longer exposure times (1 to 2 FPS) were necessary to obtain sufficient fluorescent contrast between adenomas and normal colorectal mucosa. Escalation of the bevacizumab-800CW dose up to 25 mg markedly improved the fluorescent contrast of smaller adenomas, enabling real-time visualization of all adenomas at video rate (Figure 3B and Supplemental Videos). Moreover, we were able to visualize small adenomas (<3 mm) present in the background of bright fluorescent adenomas (Figure 3B – second row). Normal colorectal mucosa showed only minimal fluorescence, resulting in a clear delineation of the fluorescent adenomas. We did not observe false positives, as no normal-appearing colorectal tissue during HD inspection showed significant NIR fluorescence with NIR-FME. In a few cases, where bowel preparation was insufficient, we did observe a detectable amount of NIR fluorescence in remaining feces. This NIR fluorescence is probably due to the native fluorescence present in fecal remnants, most likely originating from unrefined chlorophyll-containing ingredients like spinach.

**MDSFR/SFF Spectroscopy: Fluorescent Signal Quantification**

In total, the intrinsic fluorescence intensities were determined of 39 adenomas and 27 normal colon biopsies. This revealed a median adenoma-to-normal ratio for the 25 mg dose cohort of 1.84. There was a 40% increase of intrinsic fluorescence of adenomas from the 25 mg cohort compared to the 10 mg cohort (Figure 4). In contrast, the intrinsic fluorescence intensities of normal tissue remained constant for all dose cohorts.

The correction factor to correct the raw fluorescence for tissue optical properties ranged between 1.65 and 3.57. Tissue absorption, mainly by hemoglobin, was the main actor in this, while differences in scattering made a smaller but still significant contribution. The resulting intrinsic fluorescence spectra resembled the emission spectrum of bevacizumab-800CW in PBS, which confirms that the measured fluorescent signals are tracer derived (Supplementary Figure 2).

Based on the intrinsic fluorescence determined with MDSFR/SFF spectroscopy, an estimation could be made of the tracer concentration present in the tissue. This showed a median bevacizumab-800CW concentration 4.81 nmol/mL in the 10 mg adenomas, compared to 6.86 nmol/mL in the 25 mg adenomas. The median tracer concentration in normal tissue was 3.82 nmol/mL (10 mg cohort) vs 3.73 nmol/mL (25 mg cohort). These quantified measurements confirm our *in vivo* NIR-FME results, in which we observed improved fluorescence visualization of adenomas in the 25 mg dose cohort.
Figure 3. In vivo NIR-FME images. (A) Representative NIR-FME images demonstrating the visualisation of adenomas in all three tracer-dose cohorts. The first column shows the HD endoscopic image, the second and third columns show the NIR-FME images. (B) Representative video rate NIR-FME images derived from adenomas from the 25 mg dose cohort. This second series of images demonstrates the ability of the NIR-FME system to visualise even small and flat adenomas at video frame rate (10 frames per second). HD, high definition; NIR-FME, near-infrared molecular fluorescence endoscopy. Arrows indicate small and flat lesions. HD, high definition; NIR-FME, near-infrared molecular fluorescence endoscopy.
Red-Flag' Imaging Technique: Identification of Colorectal Adenomas with Wide-Field Near-Infrared Fluorescence Endoscopy

Figure 4. Fluorescence quantification by MDSFR/SFF spectroscopy. Box plot (median, 10-90 percentile) showing the intrinsic fluorescence \( (Q_{\mu f}^a) \) per bevacizumab-800CW tracer-dose cohort for both LGD containing adenomas and the normal colorectal tissue (biopsies). For all dose cohorts, a significant difference in fluorescence intensity can be observed between the benign and premalignant tissue, which increases with increasing tracer dosages. Note that the fluorescence in the normal tissue stays constant in the 10 and 25 mg cohorts, regardless of the tracer dose used. The median adenoma-to-normal ratio of intrinsic fluorescence was 1.84 for the 25 mg cohort. * = p<0.05; ** = p<0.001. MDSFR/SFF spectroscopy, multi-diameter single fibre reflection and single fibre fluorescence spectroscopy; LGD, low-grade dysplasia.

Ex Vivo Tissue Analyses: Fluorescent Signal Qualification

In total, 49 FFPE adenomas (4.5 mg n = 21; 10 mg n = 11; 25 mg n = 17) and 24 normal tissue biopsies (4.5 mg n = 8; 10 mg n = 4; 25 mg n = 12) were analyzed. Of the 49 FFPE adenomas, 48 contained LGD and one showed HGD. Within the adenomas the tracer was mainly localized in the dysplastic areas compared to the normal tissue within the adenoma section (Figure 5A). NIR fluorescence was localized between the adenomatous crypts, within the stromal tissue (Figure 5B). Normal colorectal tissue within the sections of adenomas and in the normal mucosa biopsies both showed negligible NIR fluorescence (Figure 5C).

Target-validation: VEGFA Immunohistochemistry

We observed a clear difference in VEGFA expression levels between the dysplastic crypts and the normal colon crypts (Figure 6). All adenoma samples expressed VEGFA, of which 96% showed a high staining intensity and 4% an intermediate staining (mean H-score: 286). In contrast, the normal colon tissue showed a lower mean intermediate H-score, namely 123 for normal crypts within the adenoma sections, versus 174 for biopsies of normal mucosa.
Figure 5. *Ex vivo* fluorescent signal analyses. (A) Representative NIR fluorescence flatbed scan of a fluorescent adenoma (10 mg dose cohort), containing both dysplasia and normal colon crypts in the same section (HE staining). The fluorescence scan and interactive surface plot demonstrate that the fluorescence intensities are the highest at the sites of dysplasia, a phenomenon that was observed in all three tracer-dose cohorts. (B) Three representative fluorescence microscopy images, demonstrating an observable difference in fluorescence intensities between the three dose cohorts: the 4.5 mg cohort shows a lower signal in the 800 nm channel, compared to the two higher dose cohorts. Fluorescence microscopy did not show a clear difference between the two highest dose cohorts (10 vs 25 mg). The left column represents the fluorescence of the tracer (800 nm channel), the middle column shows Hoechst staining of the nuclei and third column displays an overlay of the previous two channels. (C) Representative microscopy images of one adenoma of the 25 mg dose cohort, showing a clear difference in NIR fluorescent signal between areas containing dysplasia and areas containing normal colon crypts; the areas can be distinguished based on the appearance of the crypts, since stacking of the nuclei is typical for dysplasia. HE, haematoxylin and eosin; LGD, low-grade dysplasia.

**Figure 6. VEGFA immunohistochemistry results.** (A) Box plot (median, 10-90 percentile) and bar graph, both presenting VEGFA IHC results (H-score) of adenomatous colorectal polyps (LGD and normal crypts) and normal colorectal biopsies; a clear difference in H-score can be observed between the adenomatous crypts and the normal surrounding tissue and normal biopsies. (B) Representative images illustrating the clear difference in VEGFA staining intensities (brown) between dysplastic and normal crypts (areas within dashed yellow lines display normal crypts). VEGFA, vascular endothelial growth factor A; LGD, low-grade dysplasia.
DISCUSSION

This study demonstrates that colorectal adenomas can be identified with VEGFA-targeted NIR-FME with concurrent standard HD white-light endoscopy. Based on the real-time observations in FAP patients, we identified 25 mg bevacizumab-800CW as the best-performing tracer dose. With this dose, we were able to identify all small (<5 mm) adenomas at video-rate, even if those were situated in the background of the image, behind a larger fluorescent adenoma. Detailed ex vivo fluorescence signal analyses with MDSFR/SFF spectroscopy and microscopy confirmed the specificity of the obtained results. As such, we believe that VEGFA-targeted NIR-FME has the potential to improve real-time, wide-field colorectal adenoma identification and could therefore be a promising ‘red-flag’ identification-tool during colonoscopy and thus assist the endoscopist in adenoma detection.

Previously described targeted FME approaches have been mainly restricted to preclinical application; they described a wide range of molecular targets, fluorescent labels and administration routes, as well as the use of both wide-field macroscopic and microscopic imaging systems. Clinically, only two promising studies on wide-field FME application for colorectal adenoma detection have been published thus far. The cMET study showed positive results, though fluorescence imaging was compared with outdated fiber white-light detection, no reflection of clinical practice. Joshi et al. demonstrated specific binding of a small fluorescent peptide to sessile serrated adenomas in the proximal colon after topical administration, though, all lesions were first detected by white-light endoscopy.

Our FME approach is distinctive from both approaches as it provides instant red-flag identification of colorectal lesions. The fluorescence and white-light images could concurrently be captured, as bevacizumab-800CW emits fluorescent light outside of the visible light spectrum; in the NIR light spectrum. Hence, the fluorescent signals could instantly be superimposed on the white-light images shown to the endoscopist. The clear discrimination between colorectal adenomas and normal tissue is a result of the accumulation of the IV injected bevacizumab-800CW specifically in colorectal adenomas, and the negligible attribution of human autofluorescence in the NIR light spectrum. Future software improvements could automatically alert the endoscopist and thereby reduce the human factor.

Most likely the optimal tracer dose used during NIR-FME will have to be determined per tracer, organ of interest and indication. The aim of this study was to identify the best dose of IV bevacizumab-800CW for colorectal adenoma identification purposes. In cancer lesions it is thought that, due to the enhanced permeability and retention (EPR) effect, antibodies will accumulate in cancer, even when only a microdose of tracer is used. However, in case of precancerous lesions, such as adenomatous polyps in which vascular hyperpermeability
is not yet present, a higher tracer dose may be required to ensure sufficient fluorescent signal strength.

To identify the optimal dose, adequate fluorescent signal quantification is essential since it validates the observed in vivo fluorescent signals. The background tissue optical properties, i.e. absorption and scattering, make quantitative measurements on in vivo NIR-FME images complicated. MDSFR/SFF spectroscopy can correct raw fluorescent signals for the influence of the local tissue optical properties and was previously validated in silico and in optical phantoms, and has been used in in vivo pre-clinical models.\textsuperscript{21-24} The spectroscopy results showed 25 mg as the best-performing tracer dose, with an increase in adenoma-to-normal ratio with escalating tracer dosages. This applied bevacizumab dose of 25 mg is still far below the conventional therapeutic dose of 5-10 mg/kg and did not show any side-effects.\textsuperscript{11} In addition, we subsequently calculated the actual bevacizumab-800CW concentration in the tissue with the assumption that the in silico measured fluorescence quantum yield and extinction coefficient of bevacizumab-800CW are representative for the in vivo conditions.

Although our results are promising, our proof-of-principle study has some limitations. First, since small groups were used per tracer cohort, this study design cannot address the effect of NIR-FME on the adenoma detection rate. Secondly, FAP patients are not an ideal patient population for evaluating adenoma detection miss rates, since they are known to have multiple areas with aberrant crypt foci, and most patients have an ileorectal anastomosis. Nevertheless, we deliberately performed this proof-of-principle study in FAP patients to ensure sufficient adenoma numbers to identify the optimal tracer dose, despite the limited number of available patients. As we previously showed 79-96\% overexpression of VEGFA in adenomas of LS patients and 94\% overexpression in SSA/Ps, bevacizumab-800CW might be universally used to detect high-risk lesions.\textsuperscript{8}

In conclusion, this study demonstrates that VEGFA-targeted NIR-FME is a promising optical molecular red-flag imaging approach for colorectal adenoma detection: our novel probe-based NIR-FME approach meets the current clinical standards since it provides real-time molecular guidance, and thus functional information, without interfering with the regular HD morphological information of white-light endoscopy. Following IV administration of 25 mg of bevacizumab-800CW, NIR-FME highlighted very small dysplastic adenomas with high in vivo fluorescent contrast at video-rate, which was confirmed by MDSFR/SFF spectroscopy. Future studies are required to determine the effect of NIR-FME on the adenoma detection rate and the clinical benefit for patients at high risk of developing colorectal cancer, for example patients with Lynch syndrome.
ACKNOWLEDGEMENTS

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COMPETING INTERESTS

GMvD and WBN received an unrestricted research grant made available to the institution for the development of optical molecular imaging from SurgVision BV (’t Harde, the Netherlands). GMvD and VN are members of the scientific advisory board of SurgVision BV.
REFERENCES


SUPPLEMENTAL MATERIALS

NIR fluorescent tracer: bevacizumab-800CW
Tracer production and administration. Clinical grade bevacizumab-800CW was produced at the University Medical Center Groningen (UMCG, Groningen, the Netherlands), according to good manufacturing practice (GMP) guidelines. The labelling of the monoclonal antibody bevacizumab (Avastin, Roche, Hertfordshire, United Kingdom) with the near-infrared (NIR) fluorophore IRDye 800CW (IRDye800CW-NHS ester; LI-COR Biosciences, Lincoln, NE, USA) was performed in a 4:1 or 2:1 dye-to-protein [D:P] molar ratio in a phosphate-buffered saline (PBS, pH 8.5) solution (Supplementary Table 1). The D:P molar ratio was lowered from 4:1 to 2:1 to guarantee stability of tracer compound. The lower ratio was administered in the 10 mg and 25 mg groups.1,5

Supplementary Table 1. Tracer production characteristics

<table>
<thead>
<tr>
<th>Dose group</th>
<th>D:P*</th>
<th>Labeling efficiency (%)</th>
<th>Theoretical D:P</th>
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<tr>
<td>4.5 mg</td>
<td>4:1</td>
<td>80 %</td>
<td>2.0:1</td>
</tr>
<tr>
<td>10 and 25 mg</td>
<td>2:1</td>
<td>85.4 %</td>
<td>(20-65):1.67:1</td>
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*Dye-to-protein molar labelling ratio

Near-infrared fluorescence molecular endoscopy (NIR-FME)
Probe-based NIR-FME system. The NIR-FME system (Surgvision, ’t Harde, the Netherlands) is developed in such a way that it can be easily incorporated in clinical endoscopy procedures. It is a probe-based system, making use of a custom-made Micrendo® fiber bundle, containing 30,000 coherently-arranged individual fibers (Schölly Fiberoptic GmbH, Denzlingen, Germany). The fiber bundle has a field of view of 89° and a diameter of 2.4 mm, making it suitable for insertion through the working channel of a commercially available video endoscope. White-light illumination of the fiber was provided by a LED light source (KL 2500 LED, Schott AG, Mainz, Germany) with a shortpass dichroic filter (E7005P-SP; Chroma, Bellows Falls, VT, USA). Fluorescence excitation was achieved by two class IIIb lasers (750 nm, max. power 300 mW; BWFi, B&WTEK, Newark, DE, USA). Both light sources are coupled to the fiber bundle via a multi-branched fiber-optic bundle (SEDI-ATI Fibres Optiques, Courcouronnes, France). The fiber bundle itself is connected to a mechanical and focusing adapter (Schölly Fiberoptic, Denzlingen, Germany). This connection conducts the fiber images via a dichroic mirror and bandpass filter (819 nm, ±44 nm, Semrock Inc, Rochester, NY, USA) to a charge-coupled digital (EM-CCD) camera, sensitive for NIR light, and a separate camera for color detection (Supplementary Figure 1A). The cameras are installed at a movable arm with two swivel joints. The software generates an overlay image of both cameras and projects this real-time during the procedure on a second screen (Figure 2). In this way, fluorescence molecular guidance is realized with little impact to current workflow of clinical endoscopy procedures. The performance of the NIR-FME system was characterized as described before, with a spatial resolution of 198.42 µm at a distance of 2 cm, and a detection limit at a concentration of 19.80 nM.3,10

MDSFR/SFF spectroscopy
Instrument description. The Multi Diameter Single Fiber Reflectance and Single Fiber Fluorescence (MDSFR/SFF) spectroscopy device uses an optical fiber probe, consisting of two adjacent optical fibers with different diameters (0.4 and 0.8 mm). White-light from a tungsten halogen lamp (HL-2000-FHSA; Ocean Optics, Duiven, NL), was directed through these fibers, for the sequential acquisition of a reflection measurements using two spectrometers (SD-2000, Ocean Optics). Subsequently, 775 nm laser light was directed through the 0.8 mm fiber to obtain a fluorescence spectrum using a separate sensitive spectrometer (QE-65000, Ocean Optics). A long pass filter (785 nm) was used to block scattered
excitation light (Supplementary Figure 1B). The tip of the probe has an angle of 15° to minimize internal specular reflections. Spectrometers and light sources were controlled by a custom-made LabView program (LabView 7.1; National Instruments Corporation, Austin, TX, USA) as described previously.8,9 Before every procedure, a calibration was performed to correct for fiber alignment and transmission efficiency, using a 1.3% intralipid phantom.10 An ex vivo MDSFR/SFF spectroscopy measurement took approximately 3 sec per location.

**Spectral fitting and the determination of intrinsic fluorescence.** The MDSFR/SFF spectroscopy device was used to quantify the NIR fluorescent signals of the freshly resected colorectal tissue. The approach is based on the measurement of multiple, in the case of the current study, two single fiber reflectance spectra. From these spectra the wavelength dependence of the reduced scattering coefficient μₛ and the parameter Y (λ), which is related to the angular distribution of light scattering in tissue, is determined. It is then possible to determine the absorption coefficient μₐ(λ) of the tissue based on a set of specific chromophores: bilirubin, oxygenated hemoglobin and deoxygenated hemoglobin. By combining the measurements of the tissue optical properties (μₛ and μₐ) at both the fluorescence excitation wavelength (775 nm) and the fluorescence emission wavelength range of the tracer (780 – 850 nm) with a measurement of the raw fluorescence from the tissue, it is possible to recover the intrinsic fluorescence Qμᵣₐ. The intrinsic fluorescence Qμᵣₐ is defined as the product of the quantum efficiency across the emission spectrum, Q[·], where Q is the fluorescence quantum yield of IRDye 800CW and μᵣₐ (mm⁻¹) is the tracer absorption coefficient at the excitation wavelength.

The methodology of the determination of the correction factor has been previously described in detail.10 Quantitative fluorescence spectra were fitted as a linear combination of background autofluorescence basis spectra, determined from adenomatous polyps of a FAP patient that had not received the fluorescent tracer and average basis spectra formed from two patients that received the highest dose of tracer (25 mg). To overcome the problem of noise in the fluorescence signal between 780 and 790 nm (where our detection filter blocks both scattered excitation and fluorescence light) and to satisfy our intention to include the whole of the emission spectrum in the calculation of Qμᵣₐ, we utilized a spectrum of bevacizumab-800CW in PBS to fit spectral data below 790 nm.

Supplementary Figure 2A and 2B show the measured reflectance spectra of two LGD adenomas, both derived from the 25 mg dose cohort. In adenoma A the estimated optical properties are μₛ(775 nm) = 2.53 mm⁻¹ and μₐ(775 nm) = 0.0004 mm⁻¹ and of adenoma B μₛ(775 nm) = 0.61 mm⁻¹ and μₐ(775 nm) = 0.0087 mm⁻¹. The characteristic wavelength dips around 400 nm and between 500-600 nm are caused by the strong absorbance properties of hemoglobin. When comparing the reflectance spectra of both adenomas, note the significantly higher reflectance over the whole wavelength range in adenoma A (0.8 mm diameter fiber, blue). This is because this polyp contained significantly less hemoglobin compared to adenoma B, which clearly shows the characteristic double absorption dip centered on 575 nm due to oxygenated hemoglobin. The measured raw fluorescence was subsequently corrected for the estimated optical properties to yield the intrinsic fluorescence Qμᵣₐ. Adenoma B needed a higher correction factor to yield the intrinsic fluorescence because of the higher light absorption by oxygenated hemoglobin. That the tissue optical properties differed over all tissue samples, is reflected in the correction factor that ranged from 1.65 to 3.57. Supplementary Figure 2C shows a representative example of an intrinsic fluorescence spectrum (and its fit in blue). The ex vivo fluorescence spectrum closely resembles the tissue spectrum of bevacizumab-800CW in PBS (data not shown).

**Determination of bevacizumab-800CW concentration.** In the calculations of in-tissue bevacizumab-800CW concentration, we assumed that all IRDye-800CW fluorescence originated of intact bevacizumab-800CW. With knowledge of the intrinsic fluorescence, Qμᵣₐ, it is interesting to determine an estimation of the actual concentration of bevacizumab-800CW present in the tissue. To estimate its extinction at 775 nm, we first acquired an absorption spectrum of bevacizumab-800CW (D:P molar ratio of 2:1, in 50 mM phosphate NaCl) (Supplementary Figure 2D). From this, we calculated the extinction coefficient of bevacizumab-800CW at 775 nm of approximately 311,204 M⁻¹ cm⁻¹. The best available data in the literature suggests that fluorescence quantum yield of IRDye800CW is 0.09.11 When we assume that the extinction coefficient as well as the fluorescence quantum yield are representative for the in vivo conditions, we can calculate the tissue tracer concentration by dividing the intrinsic fluorescence Qμᵣₐ, by the extinction coefficient and the fluorescence quantum yield. Under these assumptions the median measured bevacizumab-800CW concentration in LGD adenomas after the IV administration of 25mg was 6.86 nmol mL⁻¹.

**Ex vivo signal analyses.**

**NIR fluorescence flatbed scanner.** The Odyssey CLx imaging system (LI-COR Biotechnology, Lincoln, NE, USA) is a NIR fluorescence flatbed scanner. It contains a solid-state laser diode to excite at 785 nm and silicon avalanche photodiodes to detect at 800 nm, creating high quality signal analyses.

**Immunohistochemical analysis of VEGFA expression.** At start, 4 μm FFPE tissue sections were deparaffinized and rehydrated. Heat-induced antigen retrieval was performed with citrate buffer (10mM, pH 6.0, 15 min microwave), followed by endogenous peroxidase blocking with 1.5 mL 30% hydrogen peroxide in 48.5 mL PBS (30 min). After PBS washing steps, the sections were incubated overnight at 4°C with polyclonal rabbit anti-human VEGFA (RBP031; Thermo Fisher Scientific, Waltham, MA, USA, 0.2 μg/mL, dissolved 1:500 in PBS with 1% bovine serum albumin (BSA)). Tissue was stained using goat-anti-rabbit-HRP IgG (DAKO, Glostrup, Denmark, 1:50 in PBS, 1% BSA, 1% antibody serum) for 30 min at room temperature, followed by rabbit-anti-goat-HRP (DAKO, 1:50 in PBS, 1% BSA, 1% antibody serum). Visualization took place with diaminobenzidine (DAB) for 10 min. Finally, the sections were counterstained with Mayer's hematoxylin, dehydrated and mounted with a...
glass cover slip. Dysplastic crypts, normal crypts in the adenoma sections and biopsy derived normal mucosa were scored separately for their staining intensity (0-3 scale) and the percentage of cells stained. This visual scoring was performed by two observers (JT and EH). Subsequently, H-scores were generated (continuous scale: 0-500) by combining the evaluated intensity and the corresponding percentage of cells stained (formula used: \( 1x \) (percentage of cells weakly stained \( [1+] \) + \( 2x \) (percentage of cells moderately stained \( [2+] \) + \( 3x \) (percentage of cells strongly stained \( [3+] \)), creating expression categories representative for overall protein expression per tissue type (0-100 = negative/low; 101-200 = intermediate; 201-300 = high).

Supplementary figure 1. (A) Schematic representation of the NIR-FME system. Fluorescence excitation is provided by two laser sources (750 nm); fluorescence and white-light detection is simultaneously derived by a charge-coupled digital (EM-CCD) NIR camera and a colour camera. The optical fibre bundle is inserted through the working channel of a clinical HD endoscope, creating wide-field FME. (B) Schematic representation of the MDSFR/SFF spectroscopy device. Reflection spectra are measured by the two bundled optical fibres (Ø 0.4 and 0.8 mm), followed by one fluorescence spectrum measurement via the largest fibre (Ø 0.8 mm, 775 nm fluorescence excitation light). NIR, near-infrared; HD, high definition; FME, fluorescence molecular endoscopy; BPF, band pass filter; SPF, short pass filter.

Supplementary figure 2. MDSFR/SFF spectroscopy. (A-B) Two examples of MDSFR spectra measured for two resected colorectal adenomas with LGD. The percentage of reflected light is plotted against wavelength, blue for the large fibre (Ø 0.8 mm) and red for the small fibre (Ø 0.4 mm) plus standard deviations. The larger fibre measurement always shows a higher reflectance compared to the smaller fibre due to the larger collection area. Underneath, the corresponding residual lines are plotted to show the difference between the mathematical fit and the actual data. Both fibres show a higher percentage of noise in the lower and higher wavelengths. (C) A representative corrected SFF spectrum of an adenoma from the 25 mg cohort depicted in green. The measured fluorescence is here corrected for the optical properties (tissue absorbance and scattering) calculated from the measured MDSFR spectra. The large error bars around 790 nm are due to the filters applied to block the excitation light. This green spectrum resembles the fluorescence spectrum of bevacizumab-800CW in PBS, which confirms that the measured fluorescent signals are tracer derived. The blue line is the mathematical fit to the data. (D) The absorption spectrum of bevacizumab-800CW dissolved in a 50 mM Isotate NaCl solution. A protein peak is detected at 280 nm and the bevacizumab-800CW absorption peak at 775 nm. This spectrum was used to calculate the absorption coefficient at 775 nm.
SUPPLEMENTARY REFERENCES