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***In vivo* molecular mapping of the tumor microenvironment in an azoxymethane-treated mouse model of colon carcinogenesis**

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Abstract

Background and Objective—Development of miniaturized imaging systems with molecular probes enables examination of molecular changes leading to initiation and progression of colorectal cancer in an azoxymethane (AOM)-induced mouse model of the disease. Through improved and novel studies of animal disease models, more effective diagnostic and treatment strategies may be developed for clinical translation. We introduce use of a miniaturized multimodal endoscope with lavage-delivered fluorescent probes to examine dynamic microenvironment changes in an AOM-treated mouse model.

Study Design/Materials and Methods—The endoscope is equipped with optical coherence tomography (OCT) and laser induced fluorescence (LIF) imaging modalities. It is used with Cy5.5-conjugated antibodies to create time-resolved molecular maps of colon carcinogenesis. We monitored *in vivo* changes in molecular expression over a five month period for four biomarkers: epithelial growth factor receptor (EGFR), transferrin receptor (TfR), transforming growth factor beta 1 (TGF β 1), and chemokine (C-X-C motif) receptor 2 (CXCR2). *In vivo* OCT and LIF images were compared over multiple time points to correlate increases in biomarker expression with adenoma development.

Results—This system is uniquely capable of tracking *in vivo* changes in molecular expression over time. Increased expression of the biomarker panel corresponded to sites of disease and offered predictive utility in highlighting sites of disease prior to detectable structural changes. Biomarker expression also tended to increase with higher tumor burden and growth rate in the colon.

Conclusion—We can use miniaturized dual modality endoscopes with fluorescent probes to study the tumor microenvironment in developmental animal models of cancer and supplement findings from biopsy and tissue harvesting.

Keywords

endoscopy; fluorescence; microenvironment; molecular imaging; optical coherence tomography

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Introduction

Advances in optical imaging technologies for visualizing molecular expression of key proteins have enabled the study of cancer to progress from observation during surgery and biopsy toward examining the complexity of the tumor microenvironment. Understanding the tumor microenvironment and how fluctuations of proteins and signaling molecules affect cancer initiation and progression are key for early detection, monitoring, and effective treatment. Furthermore, imaging methods for acute examination of the tumor microenvironment in appropriate disease models may be used to test detection and treatment strategies and expedite clinical translation. However, technology is only now being developed for *in vivo* examination of this dynamic environment.

Colorectal cancer (CRC) is currently the second leading cause of adult cancer-related deaths. The disease exhibits great heterogeneity and its prognosis, along with that of many other cancers, would greatly benefit from better predictive biomarkers and more effective therapeutic targets.^{1–3} The A/J mouse treated with azoxymethane (AOM), a potent carcinogen that induces colon cancer, provides a developmental model for examining the early stages of CRC development.⁴ Studying the *in vivo* microenvironment in developmental models of cancers permits analysis of early changes associated with disease and subsequently may allow more effective detection, monitoring, and treatment.

One approach for investigating the complex interactions of cellular and noncellular components in the microenvironment includes *in vitro* study of cellular signaling and tumor development in three-dimensional tissue constructs.^{5–8} While microscopic evaluation of *ex vivo* tissue and *in vitro* models allows for high resolution imaging and sensitive monitoring of molecular fluctuations,^{9–12} *in vivo* models are preferred for studying the full spectrum of the disease rather than an isolated system. Therefore, different imaging systems and reporters have been explored for examining disease progression and identifying variations in the expression of key proteins in animal cancer models. Dark boxes have been used with luminescent reporters for live animal imaging,^{13–16} but these strategies are typically limited by low image resolution, limited penetration depth, and low sensitivity to small variations in protein markers. Optical imaging systems with fluorescent and nanoparticle reporters have been applied to the detection of protein biomarkers in engineered or exogenous tumors and grafts and have demonstrated improved detection sensitivity,^{17–21} but are also generally limited by low resolution and imaging depth. Miniaturization of imaging equipment and use of endoscopy can be employed for high resolution and sensitive imaging of fluorescent markers and greater accessibility to disease sites.^{22–25}

Alterations in the expression and function of extracellular and intracellular signaling molecules are commonly observed in cancer.^{26–29} Extracellular disease biomarkers represent excellent targets for diagnostic or therapeutic strategies. Epithelial growth factor receptor (EGFR), transferrin receptor (TfR), transforming growth factor beta1 (TGFβ1), and chemokine (C-X-C motif) receptor 2 (CXCR2) are protein biomarkers that have been associated with CRC development and changes in their expression can be monitored on cell surfaces or within the tumor microenvironment. EGFR has been shown to be overexpressed in colorectal cancer and a contributor to cancer initiation and progression.^{30–32} TfR

expression on cell surfaces correlates to cell proliferation and is found to be upregulated in cancer cells.^{33–35} Furthermore, the TGF β 1 signaling pathway is often found to be disrupted in colon cancer. The molecule can act both to inhibit tumor growth and promote tumor progression and has been shown to have an increased plasma level in CRC patients.^{28–29,36–37} CXCR2 and its signaling molecule interleukin-8 (IL-8) are important mediators of the inflammatory response and an increased level of these molecules within the tumor microenvironment has recently been implicated in CRC formation, progression and recurrence.^{38–40}

Here we introduce the use of a miniaturized multimodal endoscope with lavage-delivered fluorescent probes to examine dynamic changes in the tissue microenvironment in the AOM developmental mouse model of colon carcinogenesis. The dual modality endoscope is equipped with optical coherence tomography (OCT) and laser induced fluorescence (LIF) imaging modalities, which enable non-destructive, high resolution and high sensitivity imaging of the distal colon of AOM-treated mice. OCT is sensitive to morphological changes and provides information on the microstructural tissue changes in the distal mouse colon. LIF, in combination with the fluorescent contrast agents, provides sensitivity to shifts in the expression of specific proteins. We use this endoscope in combination with Cy5.5-conjugated antibodies to create a time-resolved molecular map of colon carcinogenesis. As this imaging platform is non-destructive, OCT images and LIF spectra may be collected within the same animal at multiple time points. We monitored *in vivo* changes in molecular expression over a five month period using a panel of four biomarkers: EGFR, Tfr, TGF β 1, and CXCR2. For each individual animal, *in vivo* OCT and LIF images were computationally compared over multiple time points to correlate increases in biomarker expression with adenoma development.

Materials and Methods

OCT/LIF imaging system

The endoscopic dual OCT/LIF system has previously been described in detail.^{41–42} Briefly, the illumination source for the OCT subsystem is a superluminescent diode with a 1300 nm center wavelength and 70 nm bandwidth. Cross sectional images of 2 mm depth $\times$ 30 mm length (512 $\times$ 3000 pixels) are obtained. The OCT images have an axial resolution of 8 μ m in tissue and a lateral resolution of 18 μ m. The LIF subsystem illumination source is a 633 nm wavelength He:Ne laser for excitation of Cy5.5 dye. The LIF system is not depth resolved, but rather is spectrally resolved. LIF illumination is unfocused and produces a 1.25 mm diameter spot on the tissue surface. The remitted fluorescence emission signal captured by the endoscope is then filtered using a 650–750 nm bandpass filter and relayed to a CCD-based spectrometer. The 100 nm spectrum bandwidth is collected over 220 pixels (0.45 nm/pixel) and 148 spectra are collected over the 30 mm length of the colon (about every 200 μ m). OCT and LIF images are simultaneously collected over the distal 30 mm of the mouse colon, at 8 evenly spaced rotations (45 degrees apart) around the circumference of the colon.

OCT images collected *in vivo* were used to create a two-dimensional map of adenoma in the colon, with axes representing distance from the anus and rotation. Adenoma in OCT images were identified according to the criteria previously described by Hariri et al.⁴³ Adenoma

volume was estimated from the maximal diameter measured on an OCT scan and assuming the adenoma was a sphere; tumor burden for a colon at any time point was computed as the sum of the approximated volume of all tumors within that colon. Tumor growth rate was calculated by dividing the difference in estimated tumor burden for an animal over two consecutive measurement points by the time period between those measurement points.

LIF spectra were used to create maps of fluorescent marker accumulation, which, in the case of targeted markers, indicate molecular expression in the colon. To separate the Cy5.5 signal of delivered probes from autofluorescence, a custom Matlab program was used to deconvolve the Cy5.5 emission spectrum from each LIF spectrum. The program uses polynomial division of a Cy5.5 spectrum collected from Cy5.5 dye within a healthy control colon from the LIF spectra to determine the fluorescence emission intensity resulting only from delivered probe. The resulting Cy5.5 intensity was spectrally integrated and plotted on the same grid used for tumor mapping. The distal 5 mm of fluorescence emission measurements were eliminated, as these measurements often included areas beyond the anus, and there was significant nonspecific signal stemming from fluorescent probe on animal hair and the squamous epithelium of the anus. An average fluorescence emission value for the entire colon at a given measurement time point was calculated from the Cy5.5 fluorescence emission intensity maps.

In accordance to a protocol approved by the university Institutional Animal Care and Use Committee, OCT and LIF images were collected from 24 female A/J mice at 4 week intervals over a 16 week period. Prior to imaging, mice were anesthetized with 100 mg/kg ketamine plus 10 mg/kg xylazine, delivered intraperitoneally. The distal colon was rinsed with saline and treated with 0.15 mL 0.1 wt% N-acetylcysteine, a mucolytic agent to enhance probe penetration, for 2 min. The colon was then rinsed with 6 mL saline and 0.15 mL of a Cy5.5 conjugated antibody probe (described below) was delivered via lavage for 1 hr. Each probe targeted to a biomarker (EGFR, TfR, TGF β 1, and CXCR2) was administered to at least 3 AOM-treated mice and 1 saline-treated (control) mouse (see Supplementary Data). The nonspecific probe was delivered to 3 AOM-treated mice. Following the incubation period, the distal colons of the mice were rinsed with 6 mL saline to remove unbound fluorescent agents. A thin layer of water-based lubricant was placed on the OCT/LIF endoscope, and it was inserted 30 mm inside the colon. Each mouse was treated with Cy5.5-tagged antibodies for the same biomarker at each time point.

Animal model

Twenty-four female A/J mice were used; 17 mice were treated with azoxymethane (AOM) and 7 were treated with saline (control). Starting at 6 weeks of age, AOM-treated mice were subcutaneously injected with 10 mg/kg AOM (Sigma, St. Louis, MO) dissolved in saline at 1 week intervals for 5 weeks. Similarly, saline-treated control mice were given subcutaneous saline injections of equivalent volume at 1 week intervals for 5 weeks beginning at 6 weeks of age.

Antibody conjugation

Antibodies for *in vivo* administration were obtained from Abcam (Ab30, Ab1086, Ab64715, Ab14935, Ab27478, for EGFR, Tfr, TGF β 1, CXCR2, and control IgG, respectively, Cambridge, MA). Each purified antibody was individually fluorescently labeled with Cy5.5 dye using a proprietary conjugation kit procured from Abcam (Ab102879). Briefly, 50 μ l of purified antibodies at a concentration of 1–1.5 mg/ml were combined with 5 μ l of a provided modifier reagent; the antibody solution was then combined with 100 μ g of lyophilized Cy5.5 dye. Following a 3 hour incubation in the dark and at room temperature, 5 μ l of a provided quencher reagent was added to the solution, and the solution allowed to incubate for 30 minutes or longer before use. For *in vivo* use, fluorescently labeled antibodies were individually diluted to a concentration of 75 μ g/ml using 0.9% saline USP grade.

Ex vivo imaging and tissue processing

Following the 16-week imaging period, the animals were sacrificed and the distal 35–40 mm of the colon was excised, sliced longitudinally, and flattened to expose the lumen. Colon lumens were observed for adenoma and imaged using a dissection microscope. *Ex vivo* analysis of harvested colon tissue was used for gold-standard verification of the number and location of adenoma in each colon at the last imaging time point. Fluorescence images of the colon lumen were collected using a Cy5.5 filter cube on a stereo surgical microscope (MVX10, Olympus). The colon tissue was then embedded in optimal cutting temperature compound within cryomolds and flash frozen using 2-methylbutane precooled in liquid nitrogen. Frozen colons were sectioned longitudinally (to provide tissue views comparable to OCT) using 5 μ m thick sections and collecting 2 consecutive sections every 500 μ m. One section per collection site was fixed using 10% formalin, washed with phosphate buffered saline (PBS), mounted using Vectashield, and imaged for Cy5.5 resulting from *in vivo* probe accumulation using an inverted fluorescent microscope (IX 71, 40x objective, mercury lamp, Cy5.5 filter cube, Olympus). The corresponding slice was DAB-stained for the same biomolecule as the *in vivo* probe target according to the procedure below.

Immunohistochemistry

To determine the immunohistochemical expression of EGFR, Tfr, TGF β 1, or CXCR2 in cryosectioned colon tissue, commercial primary antibodies for the four biomarkers were obtained from Abcam (Ab2430, Ab102697, Ab92486, and Ab14935, respectively). Sections were fixed using precooled acetone, washed in PBS, incubated with 0.3% H₂O₂ in PBS for 10 min, washed in PBS, and incubated with 10% fetal bovine serum for 1 hr at room temp. Sections were then washed with PBS again and incubated with the appropriate dilution of primary antibody with 0.5% bovine serum albumin in PBS overnight at 4°C. Following incubation, slides were washed, incubated with a biotinylated anti-rabbit secondary antibody for 30 min at room temperature, washed, incubated with the pre-diluted Sav-HRP conjugates for 30 min at room temperature, washed, and developed using DAB substrate solution. Lastly, slides were dehydrated and mounted using permount. DAB IHC slides were imaged using a 20x objective on a noninverted microscope equipped with a color camera.

Data analysis

As OCT and LIF spectra were simultaneously collected, tumor maps derived from OCT images can be compared to fluorescence emission intensity maps derived from associated LIF spectra (Figure 1). For quantitative analysis, tumor and fluorescence emission intensity maps were converted into 5×8 matrixes by dividing each of the eight scans into 5 mm lateral segments. For tumor maps, segments were considered diseased if an adenoma was located on that segment; conversely, segments were considered healthy if no adenoma were located on them. For fluorescence emission intensity maps, the average intensity over the segment was calculated. The sensitivity and specificity of the fluorescent probes to locations of adenoma were then determined for a range of fluorescent intensity thresholds to create receiver operating characteristic (ROC) curves for probes specific to each of the four biomarkers and nonspecific probe. Sensitivity was calculated as the number of true positives counted over the total number of disease segments. A segment was considered a true positive if the fluorescence emission intensity exceeded the threshold in a segment with an OCT-identified adenoma, or a segment immediately adjacent to an adenoma. This criteria was chosen due to the tendency of adjacent tissues to fluoresce strongly, possibly reflecting the effect of an adenoma on immediately adjacent tissue. Sensitivity was calculated by dividing the number of true negatives by the number of healthy segments, as determined from OCT. A healthy segment was considered a true negative if its corresponding intensity segment had a value below the threshold. The area under the curve (AUC) was calculated for each ROC curve.

Tumor maps at time points 2–4 were also compared to fluorescence emission intensity maps from earlier time points. To perform comparison over time points, slight variations in endoscope rotation relative to colon tissue imaged at the different time points had to be accommodated. A cross-correlation between tumor maps obtained at different time points was performed, allowing the rotation axis to vary. If a rotational shift produced a higher cross-correlation value, corresponding fluorescence emission intensity maps were then shifted similarly. This rotational shift was the only method used to account for variation in tumor location arising from probe insertion and tissue stretch. Lateral and torsional shifts and stretching of maps were not performed. Although there appeared to be some evidence of such distortions from time point to time point, we could not accurately account for these distortions with the image information obtained. Thus, correlations between time points are likely underestimates. Each tumor map was compared with the fluorescence emission intensity maps from the concurrent and all previous time points for the animal. However, maps from the first time point were not compared, as the mice often did not yet have tumors.

Results

No animals were lost as a result of contrast agent delivery or imaging procedures. One AOM-treated mouse from the EGFR-targeted group was lost after the third imaging time point due to hypothermia from a water pack valve failure; data from this mouse were not included in the analysis. Furthermore, all 17 AOM-treated mice developed tumors in the distal colon. At the completion of the imaging study, there was an average of 4.4 tumors per colon for AOM-treated mice. No saline-treated mice developed tumors. OCT allowed for *in*

vivo observation of tumor location and growth. When the use of OCT to detect adenoma is compared to that of gross tissue analysis over the 23 experimental mice at the final time point, the OCT system performed with a sensitivity of 97% and a specificity of 100% (see Supplementary Data). Examination of *ex vivo* tissue (Figure 2a and b) shows that OCT image-derived maps of tumor location provide an accurate reconstruction of tumor location on the lumen of the distal colon tissue (Figure 2a and c). Using OCT, we calculated that the final tumor burden for AOM-treated mice ranged from 28.5 to 170 mm³ and that tumor burden growth rates ranged from 0.4 to 90 mm³/week.

OCT and LIF images simultaneously collected after the lavage administration of a fluorescent contrast agent from the biomarker panel may be compared to examine the relationship between adenoma and molecular expression. LIF-derived fluorescence emission intensity maps correlated well with *ex vivo* fluorescence images (Figure 2b and d). We were able to identify and trace signal from all targeted contrast agents *in vivo*; however, agents targeted to CXCR2 demonstrated markedly lower signal than that of agents targeted to the other three biomarkers. An excellent visual correlation was found between OCT-derived tumor maps and areas of high fluorescence emission intensity (example given in Figure 2c and d).

Co-localization analysis using consecutive histological sections of flash frozen colon tissue excised directly after endoscopic imaging verifies targeting of contrast agents (Figure 3). Flash freezing of tissue following imaging preserved the accumulation of lavage-delivered contrast agent within the colon (left images). Examination of Cy5.5 fluorescence from *in vivo* agent accumulation and DAB signal resulting from IHC showed that the locations of these signals within the colon microstructure are concurrent for all four markers. It also validates previous observations that there is lower expression of CXCR2 (less signal from Cy5.5 in fluorescence image and less brown immunostaining in IHC image).

Time-serial collection of OCT and LIF images allowed for comparison between morphological and molecular tissue features over time. Figure 4 shows a representative time-resolved series of tumor and fluorescence emission intensity maps from the distal colon of AOM-treated mice that have been administered fluorescent antibodies that are targeted to TfR (a) or that are nonspecific (b). The targeted agent demonstrated a strong fluorescence signal resulting from accumulation and tended to localize at and surrounding adenoma sites. On the other hand, the untargeted agent provided a much weaker fluorescence signal and did not demonstrate meaningful localization at or around tumor sites. The series of tumor maps also demonstrated the ability of OCT to detect increases in tumor burden over time.

Comparison of the average fluorescence of the colon to tumor burden for each colon and time point (Figure 5a, c, e, g, i) revealed there is a positive correlation between these two factors for EGFR, TfR, and TGFβ1. Comparing average colon fluorescence emission intensity against tumor growth rate (Figure 5b, d, f, h, j) suggested a positive correlation for TfR and TGFβ1. Only a weak correlation was seen for CXCR2. The nontargeted agent did not demonstrate a strong positive trend or correlation with regard to tumor burden or tumor growth rate.

Co-location of fluorescence emission from probes and adenoma was compared by calculating the sensitivity and specificity of the correlation between tumor location and fluorescence emission intensity maps over a range of fluorescence emission intensity thresholds. Figure 6 shows the area under the resulting ROC curves, for comparisons at the same imaging time point (along positive diagonal), and for fluorescence emission intensity maps obtained at time points earlier than the tumor maps (filling in lower right-hand quadrant). Correlation is not given for the first OCT time point, as tumors were infrequently seen. From these grids, it is evident there is good correlation between EGFR-targeted antibody accumulation and tumor location at the same time point, as well as between OCT time point 4 and LIF time point 3 (Figure 6a). Strong correlations are seen for TfR and TGF β 1 (Figure 6b and c) when tumor locations are compared with the both concurrent and previous LIF-derived fluorescence emission intensity maps. The correlation between the tumor map at time point 2 and the fluorescence emission intensity map at time point 1 was particularly strong for TfR. We observed modest correlation when using CXCR2, especially at early time points (Figure 6d). Tumor and fluorescence emission intensity maps for the nontargeted agent lacked correlation at any time point combination (Figure 6e).

Discussion

Our nondestructive dual modality imaging system allows us to sample the entire distal 30 mm of the mouse colon and image the same mouse over multiple time points, with OCT providing sensitivity to morphological changes and LIF to targeted agents indicating molecular expression. As OCT and LIF images are simultaneously collected, this system allows us to correlate stages of disease and adenoma growth with changes in molecular expression.

Our results for the sensitivity and specificity of OCT for tumors in the distal colon (with visual inspection of excised colon as the gold standard) are in line with our previous studies⁴³ and provide us with the confidence to use OCT as an accurate tool for discerning tumors and mapping their locations over time. We successfully used the LIF function of the dual modality endoscope to examine the accumulation of Cy5.5-tagged antibodies targeted to EGFR, TfR, TGF β 1, or CXCR2. Collection of LIF spectra (as compared to just integrated fluorescence emission intensity) allowed for isolation of Cy5.5 fluorescence from tissue autofluorescence. As this capability is not afforded by traditional fluorescence microscopy, our LIF analysis of contrast agent distribution is more accurate than that of a dissection microscope (Figure 2b and d). The monitored panel of protein biomarkers was chosen due to their implication in colon cancer and was expected to demonstrate increased expression at tumor sites. Indeed, we typically observed higher accumulation for targeted antibodies around sites of adenoma for each biomarker (Figure 2).

Mucolytic and contrast agents were prepared directly prior to imaging and administered via lavage. There was possible variation in preparation, administration and efficiency of subsequent washing, which lead to some variation in expected fluorescence emission intensity. This variation may contribute to inconsistency between animals and time points. However, in general, the lavage delivery protocol worked well, with essentially no background signal from the targeted fluorescent agent seen in extra-colon tissues.

The signals from targeted fluorescent agents administered *in vivo* and from DAB immunostaining in consecutively collected colon tissue sections are concurrently located for each experimental biomarker (Figure 3). This is evidence that the accumulation of fluorescent agents for the biomarker panel was directed by the preferential binding of the fluorescent antibodies to their molecular targets, and that fluorescence intensity maps collected from LIF spectra of targeted agents may be treated as molecular maps of the colon. Untargeted fluorescent agents did not demonstrate significant accumulation or preferential targeting at or around adenoma sites (Figure 4b), providing additional evidence that accumulation of targeted agents was the result of an active binding process.

Examination of a time-resolved imaging series for targeted fluorescent agents (Figure 4a) further supports the association between tumor location and increased molecular expression of the biomarker panel. It also suggests that increased molecular expression may precede higher tumor burden, as an area of increased fluorescence emission intensity is seen in a mouse administered TfR antibodies at time point 1, in an area near where an adenoma was detected at time point 2 (Figure 4a). Close examination of the OCT image at time point 1 reveals about a 40% increase in mucosal thickness, which was not severe enough to classify as disease. High fluorescence intensity preceding regions of adenoma development was seen in colons treated with TfR, TGF β 1, and CXCR2. Between time points 1 and 2, there was an observed increase in fluorescence in 2 out of 3 AOM-treated colons with TfR- targeted antibodies and 2 of 4 AOM-treated colons with CXCR2-targeted antibodies prior to any tumor development. For TGF β 1, areas of high fluorescence preceded adenoma development in 3 out of 3 AOM-treated colons, but this occurred in colons that had already developed other adenoma.

The relationship between fluorescence and tumor development was further examined by plotting the tumor burden of an individual mouse against the average fluorescence emission intensity detected within that mouse colon (Figure 5a, c, e, g, i). EGFR, TfR, and TGF β 1 exhibit an upward trend between tumor burden and average fluorescence emission intensity. CXCR2 does exhibit some, although slight, positive correlation, while untargeted control antibodies do not demonstrate a discernable relationship between fluorescence emission intensity and tumor burden. Upward trends observed with targeted antibodies may be the result of increased biomarker expression, increased tumor volume, or a combination of these sources. In plotting average fluorescence emission intensity in the distal colon against tumor growth rate (Figure 5b, d, f, h, j), a general upward trend is observed for TfR- and TGF β 1-targeted fluorescent dyes. CXCR2 also shows some positive, yet weak, correlation. Untargeted antibody lacks an observed trend. The positive trend between EGFR-targeted fluorescence dye emission intensity and tumor burden reflects previous studies demonstrating upregulation of the receptor, but with unknown prognostic value.⁴⁴ The relationship between fluorescence emission intensity and tumor burden observed for TfR- and TGF β 1-targeted dyes supports previous findings on their role in cellular proliferation and disease progression,^{35,45–46} as expression could be highest when tumors are rapidly growing, as well as simply large. While visual observation of time-serial OCT and LIF maps for mice treated with CXCR2-targeted agents (Supplementary Data) provides evidence for a positive correlation between tumor burden and fluorescence, the low signal for this

biomarker may have skewed quantitative analysis. Overall, these data suggest that variations in expression trends can be used to discern the role of biomarkers in *in vivo* models of carcinogenesis, including relations to metabolism and growth.

In addition to the colon-averaged data presented in Figure 5, we also compared the co-localization of adenoma to high fluorescence emission intensity at different time points to examine how changes in molecular expression may precede discernible morphological changes. EGFR, TfR and TGF β 1 exhibit high correlation when comparing OCT and LIF maps collected at the same time point (Figure 6a, b, c, positive diagonal grid elements). TfR and TGF β 1 also demonstrate a strong correlation for tumor maps compared to molecular maps collected during the previous time point (grid elements to the bottom right). Of particular interest, TfR demonstrates good correlation between tumor maps collected at time point 2 and fluorescence emission intensity maps collected at time point 1, in agreement with its established role in early-stage tumor development including cellular growth and metabolism and possibly indicating that this increased expression occurs at a cellular level. CXCR2 shows a stronger correlation than nonspecific probe, but this correlation is low; as described above, this low correlation may be attributed to low signal stemming from weak expression of CXCR2. The lack of correlation for nonspecific probe further supports that observed trends are not caused by passive accumulation. While tissue growth, distortion of the colon between time points, and variation in contrast agent preparation and administration will prevent perfect correlation, this system overall exhibits a high degree of consistency that enables comparison between time points and examination of disease. System correlation may be enhanced in subsequent studies for more comprehensive *in vivo* molecular analysis by increasing the number of scans collected for each mouse colon and allowing additional degrees of freedom in cross-correlation.

The dual modality OCT and LIF system described here uniquely discerned physiologically relevant variations in biomarkers within the microenvironment of an induced cancer model without requiring biopsy or *ex vivo* analysis. Discerning the role of proteins and signaling molecules in cancer initiation and progression is a key step towards developing tools for earlier detection, minimally invasive staging and monitoring, and personalized therapeutic targeting of colorectal cancer. Furthermore, this minimally invasive study of appropriate colon cancer mouse models may be used to expedite the transition of developing chemopreventative and chemotherapeutic strategies from the benchtop to the clinic for improved patient outcomes.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Figure 1.

Data analysis and graphical summary for tumor (OCT) and fluorescence emission intensity (LIF) map time point comparisons. Tumor and fluorescence emission intensity maps were each discretized to 5x8 matrices. Sensitivity and specificity of the fluorescent marker for tumor areas were evaluated over a range of intensity values using tumor maps as truth and comparing to fluorescence emission intensity maps collected at the same or earlier time points. The areas under the resulting ROC curves (AUC) were then calculated; the numerical values converted to a grey scale, and used to fill in elements of a grid displaying correlation between tumor and fluorescence emission intensity maps over time.

Figure 2.

Images from a single AOM-treated mouse colon with lavage-delivered EGFR-targeted fluorescent antibodies, obtained at the final time point. (a) Gross tissue image of the excised colon, sliced longitudinally and opened with the lumen side up. The locations of adenoma are highlighted with black boxes. (b) Fluorescence microscope image of the same excised colon using Cy5.5 emission and excitation filters. White boxes correspond to adenoma locations shown in panel a. Scale bar on panel b applies to panels a and b and represents 5 mm. The distal colon is sampled *in vivo* with 30 mm longitudinal scans collected at 8 evenly spaced rotations; the 8 rotations are vertically combined to provide tumor location (c) and contrast agent distribution (d) from the perspective of the colon lumen. The horizontal axis is lateral location and the vertical axis represents *in vivo* rotation, with the ventral rotation at the bottom. Proximal to distal colon is oriented from left to right. (c) Representative tumor map of the colon derived from OCT images collected *in vivo*. Tumor locations are indicated in red. Space between consecutive vertical lines represents 5 mm increments. (d) Molecular map of EGFR expression derived from LIF spectra obtained *in vivo*. Intensity map represents Cy5.5 fluorescence intensity on an arbitrary units scale from 0 to 1000 (blue to red). Panels c and d are proportionately scaled.

Figure 3.

Cy5.5 fluorescence of targeted agents delivered in vivo (left) and immunohistochemical staining using DAB (right) for each of four biomarkers: EGFR(a), Tfr(b), TGFβ1(c), and CXCR2(d). Fluorescence and IHC images for each marker were collected from similar regions of the same colon. Scale bar applies to all images and represents 100 μm.

Figure 4.

Time series of OCT and LIF derived tumor and fluorescence emission intensity maps of the distal colon for AOM-treated mice administered TfR-targeted (left) or nonspecific (right) fluorescent antibodies. OCT and LIF data are collected simultaneously. The most distal 5 mm have been removed from the images as this portion was not used for subsequent fluorescence or ROC analysis. Colons were sampled at 16, 20, 24, and 28 weeks of age. Black indicates adenoma location in OCT-derived tumor maps. The intensity scale applies to Cy5.5 fluorescence emission intensity in LIF-derived maps and has an arbitrary units scale from 0 to 1000.

Figure 5.

Plots of average Cy5.5 fluorescence emission intensity of the distal mouse colon as a function of tumor burden (left column) or tumor growth (right column) for EGFR (a, b), Tfr (c, d), TGFβ1 (e, f), or CXCR2 (g, h) –targeted or nonspecific (i, j) fluorescent antibodies. Tumor burden is approximated using OCT images of the colon. For plots of fluorescence vs. tumor burden, points are collected at 16 (+), 20 (●), 24 (▼), and 28 (□) weeks of age. For plots of fluorescence vs. tumor growth, points are derived from collected data between 16 and 20 (●), 20 and 24 (▼) and 24 and 28 (□) weeks of age. Linear regression fits account

for all mice and time points of a treatment group and are represented as dotted lines. R-squared values are presented in the bottom right corner of each graph.

Figure 6.

Location correlation between tumor and fluorescence emission intensity maps for EGFR- (a), TfR- (b), TGF β 1- (c), or CXCR2- (d) targeted, or nonspecific (e) fluorescent antibodies. Tumor maps were computationally correlated to fluorescence emission intensity maps collected at the same time point as the tumor map (positive diagonal) and each previous time point (lower right-hand elements). Grey scale represents the area under the ROC curve comparing fluorescence emission intensity vs. presence or absence of disease over a range of fluorescence intensities for each agent. The grey scale applied to the area under the curve

for each tumor map/accumulation map comparison is provided in the figure, with OCT time point on the horizontal axis and LIF time point on the vertical axis. The intensity scale applies to all figures and ranges from 0 to 1. Black represents a null value.