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Abstract. The aim of this study is to evaluate the biochemical foundation and clinical capability of an image-guided near-infrared (NIR) autofluorescence (AF) spectroscopy technique for *in vivo* diagnosis of colonic malignancies during clinical colonoscopy. A novel endoscopic fiber-optic AF system was utilized for *in vivo* NIR AF measurements at 785 nm excitation. A total of 263 *in vivo* NIR AF spectra of colonic tissues were measured from 100 patients, in which 164 spectra were from benign tissue (116 normal and 48 hyperplastic polyps), 34 spectra were from precancer (adenomatous polyps), and 65 spectra were from cancer. The non-negativity constrained least squares minimization biochemical modeling was explored to estimate the biochemical compositions of colonic tissue using nine basis reference spectra from the representative biochemicals (i.e., collagen I, elastin, β-nicotinamide adenine dinucleotide, flavin adenine dinucleotide, L-tryptophan, hematoporphyrin, 4-pyridoxic acid, pyridoxal 5′-phosphate, and water) associated with structural or cellular metabolic progression in colonic precancer and cancer. High-quality *in vivo* NIR AF spectra in the spectral range of 810 to 1000 nm were acquired from colonic benign, precancerous, and cancerous mucosa under white-light reflectance endoscopic imaging guidance. Partial least squares discriminant analysis, together with the leave-one tissue site-out, cross validation on *in vivo* NIR AF spectra yields diagnostic sensitivities of 85.4%, 76.5%, and 84.6%, and specificities of 89.9%, 93.4%, and 91.4%, respectively, for classification of benign, precancer, and cancer in the colon. This work demonstrates that image-guided NIR AF spectroscopy in conjunction with biochemical modeling has promising potential for improving *in vivo* detection and diagnosis of colonic precancer and cancer during clinical colonoscopic screening.
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Keywords: autofluorescence spectroscopy; near-infrared; colon; precancer; cancer; *in vivo* diagnosis.

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1 Introduction

Colorectal cancer is one of the leading causes of cancer-related deaths, and the third most common malignancy in the world.[1] In Singapore, the incidence of colonic cancer has increased in importance over the past three decades.[2] The majority of colorectal cancers initiate from precancerous polyps.[3] Early detection and removal of precancerous polyps during colonoscopy screening is critical to effectively reduce the risk of colonic cancer development and also improve the 5-year survival rate (of up to 80% to 90%). However, identification of precancerous polyps, such as adenomatous polyps, is very challenging since the subtle macroscopic differentiation of subtype of polyps may not be apparent under a conventional white light reflectance (WLR) colonoscopy, which relies heavily on gross morphological changes of the epithelial tissue.[4] Thus, all polyps that are found during a colonoscopy are routinely resected and sent for histopathological examinations. However, the removal of polyps without malignancies could incur additional complications of perforation or bleeding, and an increase of cost and operational time during a colonoscopy.[5] In recent years, light-induced autofluorescence (AF) imaging and spectroscopy techniques that are capable of detecting the changes of morphological architectures and endogenous fluorophores of tissue have been used to complement standard white light endoscopy for improving the noninvasive, *in vivo* diagnosis of precancer and cancer in the colon.[6] Therefore, the development of an image-guided AF diagnostic technique providing *in vivo* real-time assessment of endogenous biochemicals associated with the structural matrix of tissues or cellular metabolic processes would be of significant clinical value to the identification of precancerous polyps in the colon.

In the past two decades, AF imaging and spectroscopy have been applied for diagnosis of adenomatous polyps with high detection sensitivities, especially in the ultraviolet (UV) or short visible (VIS) regions.[7–11] However, these UV-VIS studies suffer from limited penetration depth into deeper areas of tissue. Unlike UV or VIS excitation light, near-infrared (NIR) light is non-carcinogenic and has been shown to be safe for tissue diagnosis.[12–15] Both the excitation light used and the resulting tissue AF are at NIR wavelengths that can penetrate deeper into the tissue up to 1 mm.[14,15] Hence, NIR AF could potentially be useful for noninvasive *in vivo* detection of lesions located deeper inside the tissue. The NIR AF technique which takes the advantage of

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endogenous fluorophores such as collagen, nicotinamide adenine dinucleotide (NADH), and flavin adenine dinucleotide (FAD), has been explored to improve the accuracy for diagnosis and detection of malignancies in the colon.[16] But NIR AF clinical applications have been limited not only by the difficulty in capturing inherently weak tissue NIR AF signals, but also by relatively less knowledge of possible endogenous fluorophores responsible for tissue NIR AF emission.

In this work, we integrated a novel bifurcated flexible fiber-probe[17] into the NIR AF spectroscopy system to realize *in vivo* NIR AF measurements on colonic tissue under the WLR endoscopic imaging guidance. The main aim of this study was to evaluate the diagnostic ability of the NIR AF technique for *in vivo* diagnosis of precancer and cancer based on endogenous biochemical changes in tissue during a colonoscopic examination. The biochemical constituents of suspicious colonic lesions localized by the WLR endoscopic imaging are assessed through biochemical modeling [i.e., non-negativity constrained least squares minimization (NNCLSM)] on the basis reference spectra of biochemicals in colonic tissue. These intrinsic tissue NIR AF biochemical signals identified could be advantageously used to assist the physicians in the targeted biopsies of suspicious mucosal tissues at a colonoscopy.

2 Materials and Methods

2.1 *NIR AF Spectroscopy System*

The integrated NIR AF spectroscopy and wide-field imaging system [Fig. 1(a)] was developed for *in vivo* tissue measurements at endoscopy.[17] Briefly, the endoscope-based NIR AF spectroscopy system consists of a spectrum stabilized 785-nm diode laser (maximum output: 300 mW, B&W TEK Inc., Newark, Delaware), a scientific-grade spectrometer (QE65000-FL, Ocean Optics, Dunedin, Florida), and a specially designed endoscopic fiber probe for both laser light delivery and *in vivo* NIR AF spectrum collection. The fiber probe (2.5 m in length; 1.8 mm in outer diameter), which can fit into the instrument channel of a colonoscope, consists of 32 collection fibers (core diameter of 200 μm) surrounding the central light delivery fiber (core diameter of 200 μm, NA = 0.22). The distal end of the fiber probe is coated with two different types of filters: the central excitation fiber is coated with a narrow bandpass filter (centered at 785 nm, FWHM = $\pm$2.5 nm) for 785 nm excitation light transmission, whereas the surrounding collection fibers are coated with edge long-pass filters (cut off at 800 nm) for tissue NIR AF collection. At the proximal ends of the fiber probe, the excitation and emission fibers were coupled into two separate in-line filter modules: one integrated with a narrow bandpass filter (LL01–785, Semrock, Inc., Rochester, New York) for allowing the 785-nm laser to pass through while reducing the laser noise; and one integrated with an edge long-pass filter (LP02–785RU, Semrock, Inc., Rochester, New York) to further block the Rayleigh scattered laser light while allowing the tissue NIR AF light to pass into the spectrometer through a specially designed round-to-line fiber bundle adapter (28 $\times$ 50 μm, NA = 0.22) for improving the signal-to-noise ratio [of up to 7.6-fold ($\sqrt{58}$)] through vertical binning of the entire CCD for maximizing *in vivo* tissue NIR AF detection.[17] A personal computer controls the system using a custom-designed program that triggers data acquisition and background spectrum subtraction. The system acquires *in vivo* tissue NIR AF spectra in the wavelength range of 810 to 1000 nm from colonic tissue within 1 s using the 785-nm laser

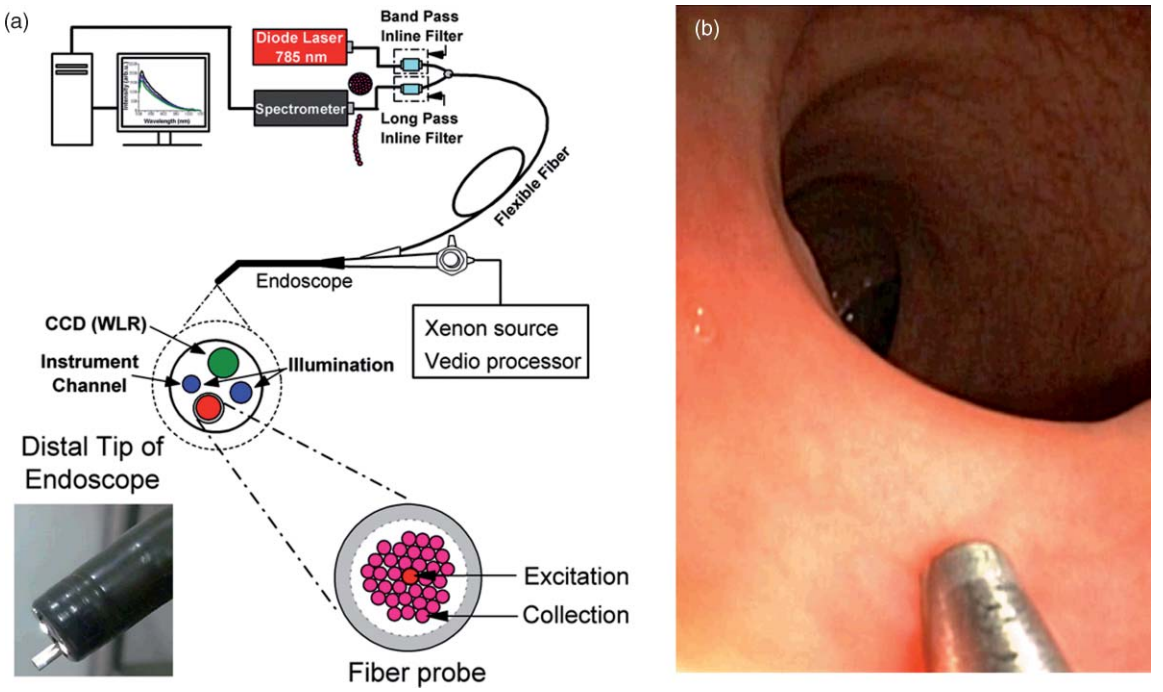

Fig. 1 (a) Schematic of the integrated autofluorescence spectroscopy and wide-field endoscopic imaging system for *in vivo* tissue NIR AF measurements at a colonoscopy. (b) WLR image of colonic tissue during clinical colonoscopy.

light irradiance of 1 W/cm^2. The wide-field colonoscope system is primarily comprised of a 300 W dedicated short-arc xenon light source, a video colonoscope (CF-Q160AL, Olympus), and a video system processor (CV-160, Olympus) for WLR imaging. With this unique NIR AF endoscopic spectroscopy system, wide-field endoscopic images and the corresponding real-time *in vivo* NIR AF spectra of colonic tissue can be simultaneously acquired, displayed, and recorded in the video system and the personal computer, respectively.

2.2 *Patients*

This study was approved by the Institutional Review Board of the National Healthcare Group of Singapore. All patients preoperatively signed an informed consent permitting *in vivo* NIR AF endoscopic spectroscopy measurements of colonic tissue during colonoscopy. Under the guidance of wide-field endoscopic WLR images as shown in Fig. 1(b), the AF endoscopic probe was directed to the suspicious lesion site, and placed gently on the colonic mucosa surface for *in vivo* NIR AF measurements. In this study, *in vivo* NIR AF spectra of 263 colonic tissue sites were acquired from 100 patients (57 male and 43 female, with a median age of 51 years) who underwent colonoscopy screening, in which 164 spectra were from benign colonic tissue (116 spectra from normal and 48 spectra from hyperplastic polyp), 34 spectra were from precancer (adenomatous polyps), and 65 spectra were from colonic cancer tissues. Immediately after NIR AF spectra acquisitions, all the measured sites with suction markings are biopsied or resected (e.g., polyps), and fixed in formalin for routine histopathological examination. For the assessment of diagnosis sensitivity and specificity of NIR AF spectroscopy technique for colonic tissue diagnosis, histopathological results serve as the gold standard.

2.3 *Biomedical Analysis*

In this study, we developed a semi-quantitative biochemical analysis algorithm based on prior biochemical knowledge using a linear combination (i.e., NNCLSM) of basis reference spectra acquired from representative biochemicals in colonic tissue.[18] The NNCLSM modeling on tissue NIR AF spectra can be expressed as

$$\text{Min}\, E(c) = \|c \cdot S - d\|^2, \quad \text{where}\, c \geq 0,$$

where c is the matrix of concentrations or contribution coefficients to be predicted, S is the matrix of spectral components of biochemicals, and d is the measured spectrum of different types of colonic tissues. This can be used to provide a "best fit" of the spectral components or basis spectra found within the measured spectrum by minimizing the difference $E(c)$ between $c \cdot S$ and d. The contribution of each basis spectrum to the *in vivo* colonic NIR AF spectra was calculated by normalizing the NNCLSM fit coefficients. The residual variations between the original AF spectra and the least-square fitting spectra presumably mainly arise from the contributions of other biomolecules that may not be fully included in the modeling.

The basis reference NIR AF spectra were obtained from the following biochemicals (Sigma-Aldrich, St. Louis, Missouri): collagen I (C9879), elastin (E1625), β-nicotinamide adenine dinucleotide (β-NADH) (N4505), FAD (F6625), L-tryphophan (T0254), hematoporphyrin (H5518), 4-pyridoxic acid (P9630), pyridoxal 5′-phosphate (82870), which represent the main endogenous components in colonic cells and tissue.[19–26] For instance, collagen I and elastin are representative of structural proteins in the extracellular matrix of the colonic wall;[20] 4-pyridoxic acid and pyridoxal 5′-phosphate represent the active form of vitamin B_6;[27] β-NADH is the co-enzyme in oxidation-reduction reactions;[28] L-tryptophan represents the amino acid that is used to synthesize protein;[29] hematoporphyrin is an endogenous porphyrin formed by the acid hydrolysis of hemoglobin;[30] FAD is the enzyme typically involved in metabolism.[7] Besides these biochemicals, deionized water was also included for spectral modeling since water is one of the main components in the tissue and cells.[31] The spectra of these nine biochemicals were measured directly from their original form (without any further purification or preprocessing) using the instrumentation previously described for semi-quantitative biochemical modeling of different types of colonic tissues.

2.4 *Multivariate Analysis*

The partial least squares discriminant analysis (PLS-DA) can advantageously be applied for multiclass classification problems by encoding the class membership of zeros and ones, representing group affinities. The PLS-DA employs the fundamental principle of principal components analysis to explain the diagnostically relevant variations but rotates the components [i.e., latent variables (LVs)] by maximizing the covariance between the spectral variation and group affinity so that LVs explain the diagnostic relevant variations rather than the most prominent variations in the NIR AF spectral dataset. In most cases, this ensures that the diagnostically significant spectral variations are retained in the first few LVs. In this study, the performance of the PLS-DA diagnostic algorithm was validated in an unbiased manner using the leave-one tissue site-out, cross validation methodology. The PLS-DA diagnostic algorithm was then used to classify the withheld NIR AF spectra. This process was repeated iteratively until all withheld NIR AF spectra were classified using the PLS toolbox (Eigenvector Research, Wenatchee, Washington) in the MATLAB (Mathworks Inc., Natick, Massachusetts) programming environment.

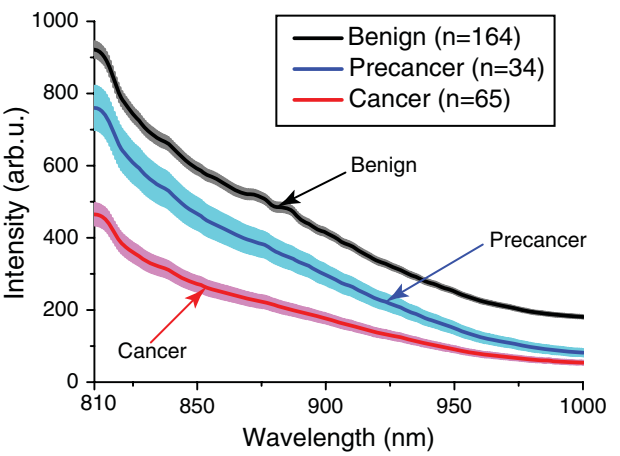

Fig. 2 *In vivo* mean NIR AF spectra ±1 standard error (SE) of benign (normal & hyperplastic polyps, $n = 164$), precancer (adenomatous polyps, $n = 34$) and cancer ($n = 65$) colonic tissue. The shaded areas in tissue NIR AF spectra stand for the respective standard errors.

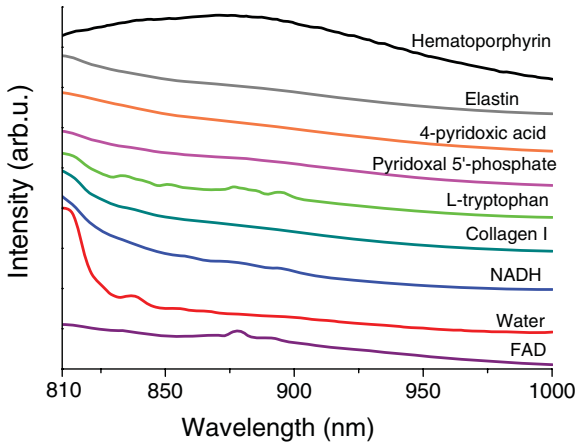

Fig. 3 The nine basis reference NIR AF spectra from collagen I, elastin, β-NADH, FAD, L-tryptophan, hematoporphyrin, 4-pyridoxic acid, pyridoxal 5′-phosphate, and water are used for biochemical modeling of the colonic tissue.

3 Results

Using the novel NIR AF spectroscopy system developed, we have successfully acquired 263 *in vivo* NIR AF spectra of colonic tissue from 100 patients under the WLR colonoscopic imaging guidance [Fig. 1(b)]. Figure 2 shows the *in vivo* mean NIR AF spectra ± 1 standard error (SE) of benign [normal ($n = 116$) and hyperplastic polyp ($n = 48$)], precancer [adenomatous polyp ($n = 34$)] and cancer ($n = 65$) colonic tissues. The significant lower AF intensity was observed in cancer tissues compared to the benign and precancer colonic tissue. The differences of fluorescence intensity among benign, precancer, and cancer tissue could be attributed to the changes of tissue optical properties in the colon,[32] such as the thickening of mucosa layer due to hyperproliferation, which could significantly attenuate the excitation light penetration and also obscure the tissue AF emission from the precancer and cancer tissue compared to benign colonic tissue.[32,33] In addition, the changes in concentrations of endogenous fluorophores such as NADH, collagen, flavins, hematoporphyrin, etc., associated with malignant transformation may also contribute to the differences in the NIR AF emission among benign, precancer, and cancer colonic tissue.

To investigate the origin of tissue biochemicals responsible for the spectral differences among benign, precancer, and cancer colonic tissues, the NNCLSM fitting procedure was employed by subjecting each tissue NIR AF spectra to the database of the nine reference spectra acquired from collagen type I, β-NADH, FAD, L-tryptophan, elastin, hematoporphyrin, 4-pyridoxic acid, pyridoxal 5′-phosphate and water (Fig. 3). Figures 4(a)–4(c) show the comparison of the reconstructed spectra with the mean *in vivo* NIR AF spectra measured for benign, precancer, and cancer colonic tissue. It is evident that acceptable fit-residuals between the reconstructed NIR AF spectra and the measured AF spectra can be achieved [residual variations: (a) benign: $\pm$2.8E-4; (b) precancer: $\pm$2.9E-4; (c) cancer: $\pm$2.18E-4], substantiating the implications of the chosen biochemicals responsible for NIR AF emission in colonic tissue. Figure 5 shows a histogram $\pm$ SE of the model fit coefficient for benign, precancer, and cancer tissue, suggesting a distinctive biochemical profile of colonic precancer compared to benign and cancer tissue. One-way analysis of variance[34] reveals that the precancer and cancer colonic tissues are associated with lower fit coefficients belonging to collagen I ($p = 1.38$E-3), FAD ($p = 2.06$E-3), β-NADH ($p = 1.12$E-12), L-tryptophan ($p = 5.21$E-14), and pyridoxal 5′-phosphate ($p = 4.08$E-2), while higher fit coefficients belonging to hematoporphyrin ($p = 1.68$E-12), 4-pyridoxic acid ($p = 3.98$E-3), and water ($p = 3.36$E-9) as compared to benign tissues. Overall, there are significant changes (i.e., increase or decrease, $p < 0.05$) in tissue biochemical constituents related to collagen type I, β-NADH, FAD, L-tryptophan, hematoporphyrin, 4-pyridoxic acid, pyridoxal 5′-phosphate, and water among the three different types of colonic tissues. The above results demonstrate that NIR AF spectroscopy is able to directly assess the biochemical changes of colonic tissue associated with cancer transformation in real-time.

To further develop effective algorithms for identifying precancer (adenomatous polyps) and cancer from benign (normal tissue and hyperplastic polyps) colonic tissue, we have employed the PLS-DA multivariate statistical technique to the standardized NIR AF spectra (each fluorescence spectrum ranging from 810 to 1000 nm with a set of 255 intensities) to determine the most diagnostically significant NIR AF features for classification of the three different types of colonic tissue. Prior to data analysis, the constructed dataset was mean centered to remove common variances. The first nine LV loadings (accounting for the NIR AF spectral variance of up to 92.5%) were found to be diagnostically significant for tissue diagnosis. Figure 6 shows a ternary plot derived when nine LVs are loaded into the PLS-DA model to generate effective diagnostic algorithms for tissue

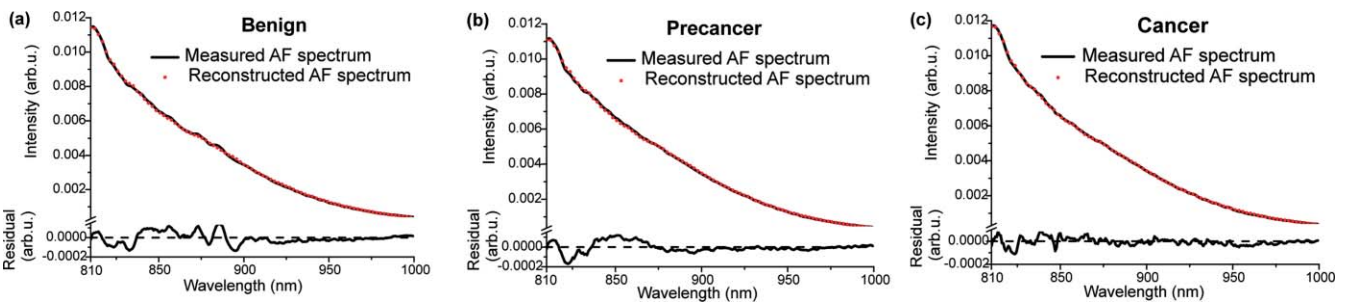

Fig. 4 Comparison of *in vivo* colonic NIR AF spectra measured with the reconstructed tissue NIR AF spectra through the employment of the nine basis reference NIR AF spectra: (a) benign, (b) precancer, and (c) cancer colonic tissue. Residuals (measured spectrum minus reconstructed spectrum) are also shown in each plot.

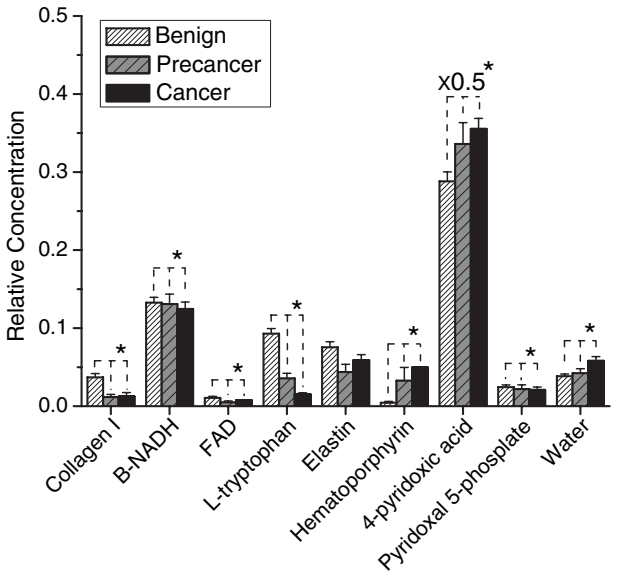

Fig. 5 Histograms displaying the average compositions of the tissues diagnosed as benign, precancer, and cancer, respectively. The one SE confidence intervals as shown for each model component. Data are clustered by model component. Note: (*) indicates the significant difference for discrimination between the three different types of colonic tissues ($p < 0.05$). The relative contribution of 4-pyridoxic acid in tissue has been reduced by a half time for better visualization in the plot.

classification. It depicts a probabilistic outcome in association with NIR AF data for each tissue type, providing a three-class diagnostic model for classification. The final diagnostic category of each data point was determined by the nearest proximity of data to the diagnostic category related to the vertex of the ternary plot. The vertices in Fig. 6 represent the 100% posterior probability belonging to benign, precancer, or cancer colonic tissue. Table 1 summarizes the diagnostic results for *in vivo* NIR AF spectra using PLS-DA together with leave-one tissue site-out, cross validation method in classifying the three different types

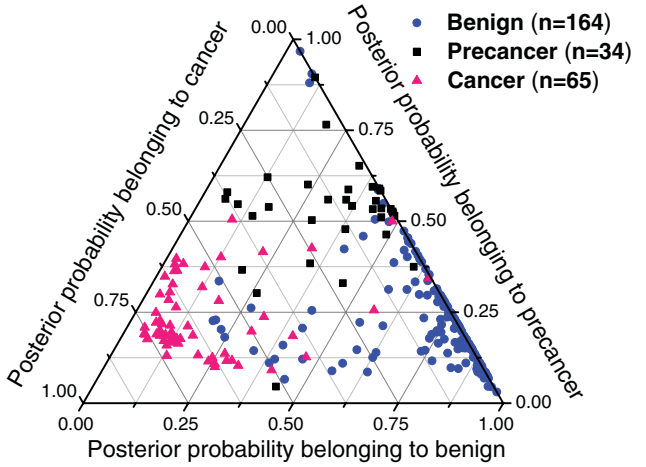

Fig. 6 Two-dimensional ternary plot of the posterior probability belonging to benign tissue, precancer, and cancer, illustrating the good clusterings of the three different colonic tissue types achieved by PLS-DA algorithms, together with the leave-one tissue site-out, cross validation method.

of colonic tissue. Diagnostic sensitivities of 85.4%, 76.5%, and 84.6%, specificities of 89.9%, 93.4%, and 91.4%, and accuracies of 87.1%, 91.3%, and 89.7%, respectively, were achieved for differentiation between benign, precancer, and cancer colonic tissues. The high predictive accuracy reinforces the robustness of NIR AF endoscopic technique for *in vivo* diagnosis of colonic precancer and cancer.

4 Discussion

Since 95% of colonic cancers arise from adenomatous polyps, early diagnosis and accurate resection of adenomatous polyps is an effective means to preventing adenoma from progression into colonic cancer.[35] However, under conventional white-light colonoscopy procedures, adenomatous polyps can be difficult to identify from hyperplastic polyps which have very low chances to develop into cancerous colonic tissue. All polyps that are found during a colonoscopy are routinely resected and sent for histopathological examinations. But the removal of polyps without malignant changes could incur additional operational time, costs, and risks (e.g., the incidence of serious complications of perforation or bleeding), which may be as high as 10% in polypectomy while only 0.2% incidence occurs in a diagnostic colonoscopy.[5] Hence, the NIR AF technique, which can provide diagnostic information of tissue morphology structure and biochemical composition in real-time to accurately identify adenomatous polyps, could be of clinically useful means to complement conventional white light colonoscopy for improving the noninvasive, *in vivo* detection and diagnosis of malignancies in the colon. In this work, we extended the previous UV/VIS AF studies[5,9,11] to the NIR domain to investigate *in vivo* NIR AF spectral properties of benign, precancerous and cancerous colonic tissues. We also explored the potential of translating NIR AF biochemical spectral differences among benign, precancer, and cancer colonic tissue into clinically useful diagnostic algorithms for realizing *in vivo* diagnosis of precancerous lesions in the colon.

Significant NIR AF spectral differences among benign, precancer, and cancer colonic tissue were observed (Fig. 2), confirming the promising diagnostic potential of image-guided NIR AF spectroscopy. To employ the NIR AF spectral information for colonic precancer tissue detection, we further developed the biochemical modeling (NNCSLM) constructed from essential endogenous fluorophores present in colonic tissue. Through the use of the nine representative biochemicals to reconstruct the measured tissue NIR AF spectra, acceptable fittings (with residuals of <5%) of the *in vivo* colonic NIR AF spectra measured were obtained (Fig. 4). The residuals [Figs. 4(a)–4(c)] likely reflect the myriad of different biochemicals that may not be fully included in the tissue model. The biochemcial modeling was constructed for colonic precancer and cancer tissue classification based on the different biochemical constituents typically present in colonic cells and tissue. The modeling showed that collagen I and elastin NIR AF signals, which represent the structural proteins in the extracellular matrix of the colonic wall, are significantly lower for precancer and cancer tissue compared to the normal tissue. This could be attributed to the thickening of the mucosa layer due to proliferation of neoplastic cells and the replacement of the submucosa by cancerous cells.[32] Subsequently, a decrease in the fluorescence emission from

Table 1 Classification results of *in vivo* NIR AF spectra prediction for the three colonic tissue groups using PLS-DA algorithms, together with the leave-one tissue site-out, cross validation method.

	NIR AF prediction			
Tissue type	Benign (Normal + Hyperplastic)	Precancer	Cancer	Total
Benign (Normal + Hyperplastic)	140	10	14	164
Precancer	5	26	3	34
Cancer	5	5	55	65
Sensitivity (%)	85.4	76.5	84.6	
Specificity (%)	89.9	93.4	91.4	
Accuracy (%)	87.1	91.3	89.7	

the submucosa collagen and elastin could ensue.[20] These findings are in agreement with collagenase studies, which usually associate the degrading collagen cross links with a decrease in collagen fluorescence during significant tissue architectural changes.[21] Additionally, significant reduction in the β-NADH and FAD AF signals of precancer and cancer colonic tissue were also observed. This phenomenon reflects the changes of metabolic activity in cancer tissue as the nicotinamide adenine dinucleotide and flavins play an integral role in cellular metabolism. The ratio of the fluorescence of FAD to the sum of the fluorescence of FAD and NADH (also described as "redox ratio") decreased in cancer, and was found to be sensitive to the changes in the metabolic rate and vascular oxygen supply.[36] On the other hand, the NIR AF signal from hematoporphyrin, an endogenous porphyrin formed by the acid hydrolysis of hemoglobin, has been found to be higher for the precancer and cancer colonic tissue than normal tissue. The accumulation of hematoporphyrin in various types of cancer has been discovered and exploited since the 1950s.[37] Since hematoporphyrin fluorescence was higher in cancer and precancer tissue than in the surrounding normal mucosa, hematoporphyrin derivatives have been established for photodetection or photodynamic therapy of colorectal cancer.[22] Significant reduction in the L-tryptophan AF signal of precancer and cancerous colonic tissue was also observed, suggesting that a mechanism by which cancer-related immune stimulation affected the tryptophan depletion.[38] On top of these findings, vitamin B_6, whose main circulation form is pyridoxal 5′-phosphate, and 4-pyridoxic acid, a major excretion product, is important in one-carbon metabolism. One-carbon metabolism is critical for DNA synthesis and DNA methylation, both of which are potentially involved in colorectal carcinogenesis.[39] Our observations of lower pyridoxal 5′-phosphate and higher 4-pyridoxic acid contents in precancer and cancerous colonic tissue is in agreement with studies of vitamin B_6 deficiency, which are associated with risk for colonic cancer and colonic adenoma.[24,26,40] One noted that the NNCLSM modeling of NIR AF spectra in this study only served as a semi-quantitative estimation of biochemical compositions of the colonic tissue, such that the results obtained should be interpreted with caution. This is because: i. the NNCLSM modeling only includes the most essential endogenous fluorophores which are known to be associated with the structural matrix of tissues or involved in cellular metabolic processes; ii. the *in vitro* biochemical conformations may not truly reflect the *in vivo* conditions; iii. the spectral reconstruction does not take into account for the effects of tissue optical properties (e.g., tissue absorption and scattering), the nonuniform fluorophore distribution in tissue and, in particular, the depth-associated variations of NIR AF signals. To better understand the relationships between the tissue morphologic/biochemical changes and the tissue NIR AF spectra for further improving tissue diagnosis and classification, confocal NIR AF microspectroscopy should be explored on the colonic tissue *in vivo* or *in vitro*, by measuring the complete NIR AF spectra of specific tissue microstructures, or alternatively by mapping the distributions of some specific NIR AF peaks or principal components within a tissue, or even mapping the biochemical distributions at different tissue depths for association with neoplastic transformation in colonic tissue. In addition, how the absorbers (e.g., hemoglobin) in tissue attenuate the NIR excitation light, and thus attenuating tissue NIR AF emission and modulating NIR AF spectral shape should be studied further. Hence, a more complete NNCLSM biochemical modeling integrated with tissue optical properties (e.g., absorption and scattering coefficients, anisotropy, tissue thickness, etc.), and light propagation in multilayer tissue model with Monte Carlo simulations warrants further investigation. Nevertheless, within these limits, we have shown that *in vivo* estimation of colonic intrinsic biochemicals compositions can be largely realized with revealing highly representative biochemicals responsible for tissue NIR AF emission, and the fitting results correlate well with histopathological findings. Therefore, the distinctive differences in NIR AF spectra among benign, precancer, and cancer colonic tissues confirm the potential role of endoscopic image-guided NIR AF spectroscopy for *in vivo* diagnosis of colonic precancer and cancer.

5 Conclusions

We have acquired, for the first time (to our knowledge), high quality *in vivo* NIR AF spectra from benign, precancerous, and cancerous colonic tissue in real-time during clinical colonoscopic examination. NIR AF spectral analysis shows significant differences among different types of colonic tissues and provides new insights into biochemical origins of NIR AF spectroscopy for diagnosis and characterization of colonic precancer and cancer. Good classification among benign, precancer, and cancer colonic tissues can be achieved using PLS-DA modeling on *in vivo* NIR AF spectra, demonstrating the potential of NIR AF technique to be a clinical complement to conventional WLR endoscopy for improving *in vivo* detection and diagnosis of colonic precancer and cancer during clinical colonoscopic examinations.

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