

Development of a synchronous fluorescence imaging system and data analysis methods

Quan Liu¹, Kui Chen², Matthew Martin², Alan Wintenberg², Roberto Lenarduzzi²
Masoud Panjehpour³, Bergein F. Overholt³, and Tuan Vo-Dinh^{1*}

¹ Fitzpatrick Institute for Photonics, Duke University, Durham, NC 27708

² Oak Ridge National Laboratory, Oak Ridge, TN 37831

³ Thompson Cancer Survival Center, Knoxville, TN 37916

* Contact email: tuan.vodinh@duke.edu

Abstract: Although conventional autofluorescence spectroscopy, in which fluorescence emission spectra are recorded for fixed excitation wavelengths, has demonstrated good performance in tissue diagnosis, it suffers from prolonged data acquisition time and broad-band fluorescence features. Synchronous spectroscopy has been proposed to overcome the limitations of conventional fluorescence spectroscopy but has not been applied to imaging for tissue diagnosis *in vivo*. Our group has developed a synchronous fluorescence imaging system to combine the great diagnostic potential of synchronous spectroscopy and the large field of view of imaging for cancer diagnosis. This system has been tested in a mouse skin model to capture synchronous fluorescence images. A simple discriminant analysis method and a more complicated multi-variate statistical method have been developed to generate a single diagnostic image from a large number of raw fluorescence images. Moreover, it was demonstrated that the diagnostic image generated from synchronous data is comparable to that generated from full spectral data in classification accuracy.

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OCIS codes: (100.2000) Digital image processing; (100.2960) Image analysis; (110.2350) Fiber optics imaging; (120.6200) Spectrometers and spectroscopic instrumentation; (170.2150) Endoscopic imaging; (170.6280) Spectroscopy, fluorescence and luminescence.

References and links

1. I. Georgakoudi and M. S. Feld, "The combined use of fluorescence, reflectance, and light-scattering spectroscopy for evaluating dysplasia in Barrett's esophagus," *Gastrointest. Endosc. Clin N Am* **14**, 519-537, ix (2004).
2. N. Ramanujam, "Fluorescence spectroscopy of neoplastic and non-neoplastic tissues," *Neoplasia* **2**, 89-117 (2000).
3. R. Richards-Kortum, M. F. Mitchell, N. Ramanujam, A. Mahadevan, and S. Thomsen, "In vivo fluorescence spectroscopy: potential for non-invasive, automated diagnosis of cervical intraepithelial neoplasia and use as a surrogate endpoint biomarker," *J. Cell Biochem. Suppl* **19**, 111-119 (1994).
4. D. M. Harris and J. Werkhaven, "Endogenous porphyrin fluorescence in tumors," *Lasers Surg Med* **7**, 467-472 (1987).
5. T. Vo-Dinh, M. Panjehpour, B. F. Overholt, C. Farris, F. P. Buckley, 3rd, and R. Sneed, "In vivo cancer diagnosis of the esophagus using differential normalized fluorescence (DNF) indices," *Lasers Surg Med* **16**, 41-47 (1995).
6. R. R. Alfano, *Advances in Optical Biopsy and Optical Mammography* (New York, 1998).
7. S. Lam, B. Palcic, D. McLean, J. Hung, M. Korbelik, and A. E. Profio, "Detection of early lung cancer using low dose Photofrin II," *Chest* **97**, 333-337 (1990).
8. T. Vo-Dinh, "Multicomponent analysis by synchronous luminescence spectrometry," *Analytical Chemistry* **50**, 396-401 (1978).
9. T. Vo-Dinh, "Synchronous luminescence spectroscopy: methodology and applicability," *Appl. Spectrosc.* **36**, 576-581 (1982).
10. W. E. Watts, N. R. Isola, D. Frazier, and T. Vo-Dinh, "Differentiation of normal and neoplastic cells by synchronous fluorescence: Rat liver epithelial and rat hepatoma cell models," *Anal. Lett.* **32**, 2583-2594 (1999).
11. D. M. Hueber, C. L. Stevenson, and T. Vo-Dinh, "Fast scanning synchronous luminescence spectrometer based on acousto-optic tunable filters," *Appl. Spectrosc.* **49**, 1624-1631 (1995).

12. M. Uziel, R. J. Ward, and T. Vodinh, "Synchronous Fluorescence Measurement of Bap Metabolites in Human and Animal Urine," *Anal. Lett.* **20**, 761-776 (1987).
13. K. Vahakangas, G. Trivers, M. Rowe, and C. C. Harris, "Benzo(a)pyrene diol-epoxide-DNA adducts detected by synchronous fluorescence spectrophotometry," *Environ Health Perspect* **62**, 101-104 (1985).
14. A. Weston, M. L. Rowe, D. K. Manchester, P. B. Farmer, D. L. Mann, and C. C. Harris, "Fluorescence and mass spectral evidence for the formation of benzo[a]pyrene anti-diol-epoxide-DNA and -hemoglobin adducts in humans," *Carcinogenesis* **10**, 251-257 (1989).
15. P. G. Shields, S. Kato, E. D. Bowman, S. Petruzzelli, D. P. Cooper, A. C. Povey, and A. Weston, "Combined micropreparative techniques with synchronous fluorescence spectroscopy or 32P-postlabelling assay for carcinogen-DNA adduct determination," *IARC Sci Publ*, 243-254 (1993).
16. M. D. Askari, and T. Vo-Dinh, "Implication of mitochondrial involvement in apoptotic activity of fragile histidine triad gene: application of synchronous luminescence spectroscopy," *Biopolymers* **73**, 510-523 (2004).
17. L. Uma, Y. Sharma, and D. Balasubramanian, "Fluorescence properties of isolated intact normal human corneas," *Photochem Photobiol* **63**, 213-216 (1996).
18. N. Vengadesan, T. Anbupalam, S. Hemamalini, J. Ebenezer, K. Muthvelu, D. Koteeswaran, P. R. Aruna, and S. Ganesan, "Characterization of cervical normal and abnormal tissues by synchronous luminescence spectroscopy," *Proc. SPIE* **4613**, 13-17 (2002).
19. T. Dramicanin, M. D. Dramicanin, V. Jokanovic, D. Nikolic-Vukosavljevic, and B. Dimitrijevic, "Three-dimensional total synchronous luminescence spectroscopy criteria for discrimination between normal and malignant breast tissues," *Photochem Photobiol* **81**, 1554-1558 (2005).
20. P. Diagaradjane, M. A. Yaseen, Y. Jie, M. S. Wong, and B. Anvari, "Synchronous fluorescence spectroscopic characterization of DMBA-TPA-induced squamous cell carcinoma in mice," *J. of Biomed. Opt.* **11**, 14012 (2006).
21. T. Vo-Dinh, "Principle of synchronous luminescence (SL) technique for biomedical diagnostics," *Proc. SPIE*, **3911**, 42-49 (2000).
22. S. L. Zhang, C. L. Meyers, K. Subramanyan, and T. M. Hancewicz, "Near infrared imaging for measuring and visualizing skin hydration. A comparison with visual assessment and electrical methods," *J. of Biomed. Opt.* **10**, 031107 (2005).
23. S. Tomatis, M. Carrara, A. Bono, C. Bartoli, M. Lualdi, G. Tragni, A. Colombo, and R. Marchesini, "Automated melanoma detection with a novel multispectral imaging system: results of a prospective study," *Physics in Medicine and Biology* **50**, 1675-1687 (2005).
24. C. Balas, "A novel optical imaging method for the early detection, quantitative grading, and mapping of cancerous and precancerous lesions of cervix," *IEEE Trans Biomed Eng* **48**, 96-104 (2001).
25. S. C. Gebhart, R. C. Thompson, and A. Mahadevan-Jansen, "Liquid-crystal tunable filter spectral imaging for brain tumor demarcation," *Appl. Opt.* **46**, 1896-1910 (2007).
26. M. E. Martin, M. B. Wabuyele, K. Chen, P. Kasili, M. Panjehpour, M. Phan, B. Overholt, G. Cunningham, D. Wilson, R. C. Denovo, and T. Vo-Dinh, "Development of an advanced hyperspectral imaging (HSI) system with applications for cancer detection," *Ann. Biomed. Eng.* **34**, 1061-1068 (2006).
27. M. Terzaghi-Howe, "Inhibition of carcinogen-altered rat tracheal epithelial cell proliferation by normal epithelial cells in vivo," *Carcinogenesis* **8**, 145-150 (1987).
28. R. A. Johnson, and D. W. Wichern, *Applied Multivariate Statistical Analysis* (Prentice Hall, 2002).
29. G. M. Palmer, C. Zhu, T. M. Breslin, F. Xu, K. W. Gilchrist, and N. Ramanujam, "Comparison of multiexcitation fluorescence and diffuse reflectance spectroscopy for the diagnosis of breast cancer (March 2003)," *IEEE Trans Biomed Eng* **50**, 1233-1242 (2003).
30. T. Vo-Dinh, *Modern Fluorescence Spectroscopy*, E. L. Wehry, ed. (Plenum Publishing Corporation, 1981), pp. 173-176.

1. Introduction

Tissue autofluorescence may be contributed by several endogenous fluorophores such as aromatic amino acids (e.g., tryptophan, tyrosine, phenylalanine), structural proteins (e.g., collagen, elastin), nicotinamide adenine dinucleotide (NADH), flavin adenine dinucleotide (FAD), porphyrins, lipopigments (e.g., ceroids, lipofuscin), and other biological components. Conventional fluorescence spectroscopy uses either a fixed-wavelength excitation (λ_{exc}) to produce an emission spectrum or a fixed wavelength emission (λ_{emm}) to record an excitation spectrum. Autofluorescence spectroscopy using laser-induced fluorescence (LIF) with fixed-excitation or fixed-emission wavelengths has been explored for cancer diagnosis by several groups [1-7]. Although changes in fluorescence profiles using fixed excitation/emission have been reported by these researchers, the changes do not provide "unique narrow-band spectral signatures" useful for unequivocal diagnostic purposes, which did not fully exploit the diagnostic potential of fluorescence spectroscopy. Furthermore, this method requires prolonged acquisition time to collect full spectral data. Our laboratory has previously developed the synchronous fluorescence (SF) method[8, 9], which could provide a simple

way to rapidly measure fluorescence signals and spectral fingerprints of complex biological samples such as tissue. With synchronous spectroscopy, the fluorescence signal is recorded while both λ_{emm} and λ_{exc} are simultaneously scanned. A constant wavelength interval is maintained between the excitation and the emission wavelengths throughout the spectrum. As a result, the intensity of the synchronous signal I_s , can be written as the product of three functions as follows^{5,6}:

$$I_s(\lambda_{\text{exc}}, \lambda_{\text{emm}}) = k E_X(\lambda_{\text{exc}}) \cdot \sum_i FE_i(\lambda_{\text{exc}}, \lambda_{\text{emm}}) \cdot E_M(\lambda_{\text{emm}}) \quad (1)$$

where

k = a constant accounting for the measurement geometry,

E_X = excitation function that describes the probability of excitation light reaching the fluorophore,

FE_i = fluorescence efficiency of the i -th fluorophore at which excitation light at λ_{exc} is converted to fluorescent light at λ_{emm} , which is related to the absorption coefficient and fluorescence quantum yield of the fluorophore,

E_M = emission function that describes the probability of emitted light being detected.

When the wavelength interval $\Delta\lambda$ between λ_{exc} and λ_{emm} is chosen properly, the resulting spectrum will show one or a few features that are much more resolvable than those in the conventional fluorescence emission spectrum measured for a fixed excitation wavelength. For example, if the wavelength interval is chosen to be the difference between the peak absorption wavelength and the peak emission wavelength for a single fluorophore, the synchronous spectrum of a sample containing this fluorophore would show a single sharp peak associated with this fluorophore.

This method has been developed for multi-component analysis and used to obtain fingerprints of real-life samples and for enhancing the selectivity in the assay of complex chemical and biological systems [9-13]. This SF procedure simplifies the emission spectrum and provides for greater selectivity when measuring fluorescence or phosphorescence from a mixture of compounds. The SF method has been the basis for various instruments developed by our group including an acoustooptic tunable filter system [11]. Most of its early applications focused on examining *in vitro* samples, for example, analyzing benzo[a]pyrene (BaP) in human and animal urine [14], determining carcinogen-macromolecular adducts [14, 15], and measuring mitochondrial uptake of Rhodamine 123 in cells [16]. Only recently this technique started to find its use in optical diagnosis of cancers. Several groups have carried out synchronous fluorescence spectroscopy on *ex vivo* tissues, including corneas [17], cervical tissues [18] and breast tissues [19]. Diagaradjane, *et al.*, [20] have performed synchronous fluorescence spectroscopy to characterize DMBA-TPA-induced squamous cell carcinoma in mice *in vivo*. Their results demonstrated high diagnostic accuracy (> 80%) in differentiating premalignant and malignant conditions from normal tissues when synchronous fluorescence spectroscopy was used in combination with a statistical analysis method. Our group has performed measurements of a variety of skin tissues to illustrate the narrow-band features and selectivity of the SF technique for *in vivo* analysis [21]. It was found that, while the conventional fixed-excitation method produces fluorescence spectra with broad-band features, the SF technique is able to provide characteristic SF signatures for different types of skin tissues.

The motivation for this work came from two aspects. First, compared to conventional fluorescence spectroscopy, where an excitation-emission matrix (EEM) needs to be measured to fully characterize samples containing multiple fluorophores, synchronous fluorescence spectroscopy provides a tool that can detect multiple fluorophores with a much smaller number of excitation and emission wavelengths thus could rapidly reduce data acquisition time [21]. However, given that synchronous fluorescence data is only a small subset of the full EEM data, one question arises naturally, i.e. whether synchronous fluorescence spectroscopy offers the diagnostic capability comparable to conventional fluorescence emission spectroscopy for tissue diagnosis.

Second, several multi-spectral imaging systems using diffuse reflectance or fluorescence with fixed excitation wavelengths have been reported. For example, multi-spectral reflectance imaging systems have been used to measure skin hydration [22], detect melanoma [23], and to assess the alterations in the light scattering properties of cervical neoplasias after the application of acetic acid solution [24]. Multi-spectral imaging that combines diffuse reflectance and fixed-excitation fluorescence has been tried for brain tumor demarcation [25]. In contrast, there were no similar imaging systems for the synchronous fluorescence technique published. All the above cited works belong to the category of point spectroscopy. It would be a crucial step to develop a synchronous fluorescence imaging system and appropriate data analysis methods in order to maximally explore the potential of this technique in real-world applications.

To apply the synchronous fluorescence technique to an imaging modality, we have developed such a synchronous fluorescence imaging system that can be incorporated into an endoscope for the GI cancer detection. Moreover, to answer the previous question, i.e. whether synchronous fluorescence spectroscopy offers the diagnostic capability comparable to conventional fluorescence emission spectroscopy for tissue diagnosis, we have tested this system in a mouse skin model to capture both synchronous fluorescence images and full spectral images. A simple discriminant analysis method and a more complicated multi-variate statistical method have been developed to differentiate pixels corresponding to malignant tissue from pixels corresponding to normal tissue. A Matlab code was written so that the training pixels can be selected manually for a chosen classifier to learn. Then the trained classifier can automatically generate a diagnostic image indicating the condition of each pixel. Both synchronous fluorescence images and full spectral fluorescence images were analyzed using the two methods. The specificity and sensitivity of the diagnostic image obtained from synchronous data were calculated, for which the diagnostic image obtained from full spectra data were used as the gold standard. These results provided the quantitative proof for the diagnostic potential of synchronous imaging.

2. Methods

2.1 System configuration

A multi-spectral imaging system was used to take the fluorescence images of the mouse skin. The schematic of the system is shown in Fig. 1. The detail of the system has been described elsewhere [26], therefore only the main features are presented as follows. An optical-parametric oscillator (OPO) pumped by a Nd:YAG laser (Opolette, Opotek, CA) provided excitation light from 420 to 700 nm with an average power of 40 mW. The laser pulses were generated at 20 Hz, and each pulse lasted for 5 ns. A quartz fiber was used to deliver the excitation light onto the mouse skin through the small biopsy channel of the endoscope and a fiber imaging bundle (# K02520AH1, Karl Storz Optical Co.) was employed to collect fluorescence light and backscattered excitation light through the large biopsy channel of the endoscope. The illuminated area (about 1 cm in diameter) was significantly larger than the imaging area to ensure uniform illumination. A beam splitter was used to deflect 7% of collected light to a charge-coupled device (CCD) to record reflectance images. The remaining 93% of collected light was allowed to transmit to a liquid crystal tunable filter (LCTF for visible spectrum with 7-nm bandwidth, Cambridge Research & Instrumentation, Inc., MA). The out-of-band transmission ratio of the LCTF is typically 0.01% according to the manufacturer's specification. The filter was tuned to only allow fluorescence light at one wavelength a time to reach an intensified CCD (ICCD) (PIMAX-512-T-18 Gen. II, Roper Scientific) to record fluorescence images.

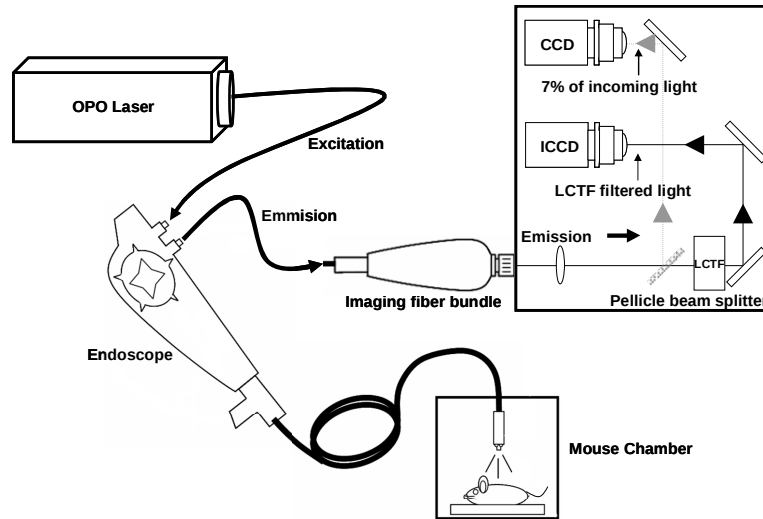


Fig. 1. Schematic of the multi-spectral imaging system.

2.2 Experimental protocol

Five nude mice, which didn't have body hair due to genetic mutation, were injected subcutaneously in the lower right and left lumbar region with 100 μ l of Fischer rat 344 tracheal carcinoma cells (IC-12) [27] at a concentration of 10^7 cells/ml to induce tumors. The mice were then incubated for seven to eight days to allow tumors to grow to approximately 5 mm in diameter. Spectral imaging data were acquired from anesthetized mice. The laser source worked at 20 Hz and the ICCD accumulated four frames for each image to improve the signal-to-noise ratio. So it took 0.2 sec to acquire one image. It took about half an hour to acquire a whole set of fluorescence images from each mouse, which included the time of manual operation as the system was not fully automated. The mice were euthanized after data acquisition.

The excitation wavelength was varied from 420 to 600 nm every 20 nm. Fluorescence images were acquired at the emission wavelength starting from the excitation wavelength plus 30 nm till 650 nm at a 10-nm increment. The image taken at ($\lambda_{\text{exc}} = 600$ nm, $\lambda_{\text{emm}} = 630$ nm) was saturated potentially due to unexpected ambient light thus not used in analysis. This procedure yielded totally 119 images as shown in Fig. 2. The synchronous fluorescence images were a subset of the fluorescence images, for which the excitation wavelength varied from 420 to 600 nm every 20 nm. The single emission wavelength was the excitation wavelength plus $\Delta\lambda$ (differential wavelength), which yielded totally 8 to 10 images. The differential wavelength was varied from 30 to 90 nm every 10 nm to obtain seven sets of synchronous data. As an example, the images marked by dashed rectangles in Fig. 2 are the synchronous data for $\Delta\lambda = 30$ nm.

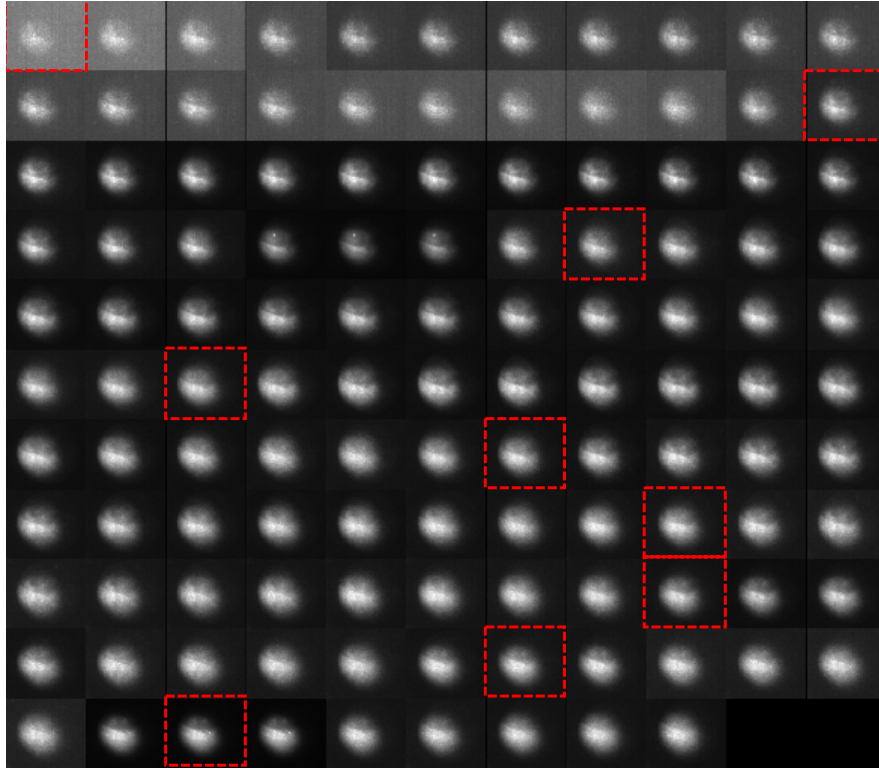


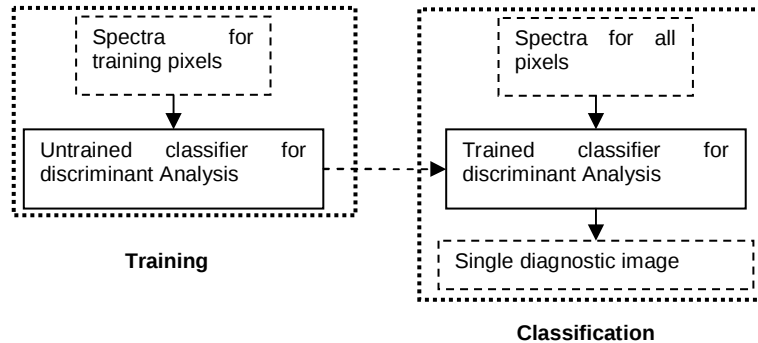
Fig. 2. Panel of raw fluorescence images. In the order from left to right and then from top to bottom, the initial excitation wavelength is 420 nm and the emission wavelength starts with the excitation wavelength plus 30 nm and increases at an increment of 10 nm first. When the emission wavelength reaches 650 nm, the excitation wavelength increases by 20 nm and the emission wavelength starts from the new excitation wavelength plus 30 nm and increases again. This continues till ($\lambda_{\text{exc}} = 600$ nm, $\lambda_{\text{em}} = 650$ nm). The image taken at ($\lambda_{\text{exc}} = 600$ nm, $\lambda_{\text{em}} = 630$ nm) was not shown because it was saturated. As an example for synchronous data, the images highlighted by the dashed rectangles correspond to synchronous data acquired for $\Delta\lambda = 30$ nm. It should be pointed out that the absolute intensity levels of those images have been changed from their original values to ensure the visibility of all the images in one panel.

2.3 Data analysis

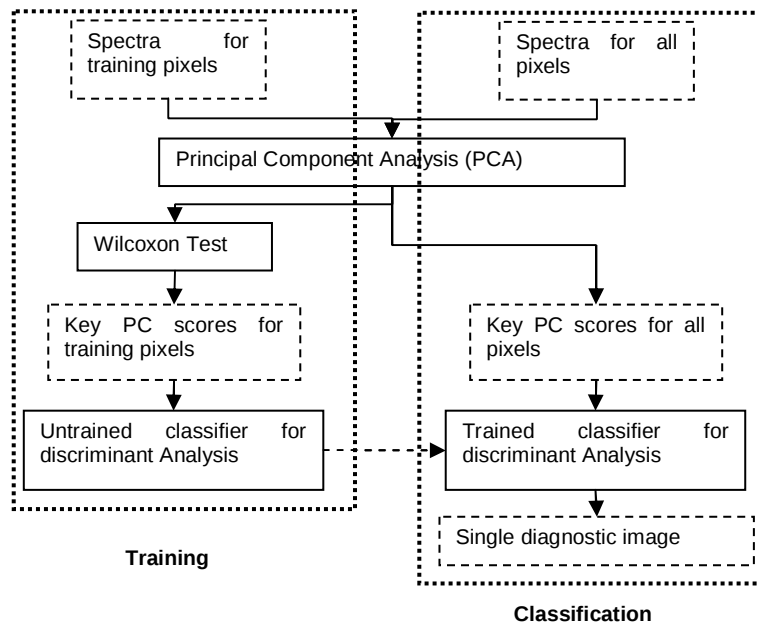
All the fluorescence images were first normalized by the output power of the OPO light source at the corresponding excitation wavelength to remove the wavelength dependence of the excitation power. The second step of preprocessing is to remove motion artifacts that were likely due to animal breathing. To remove the motion artifacts, the normalized intensity images were converted to binary images by assigning one to any pixel whose intensity is larger than the average of the 25-th and 75-th percentiles in the set of all pixel intensity values and zero to other pixels. The centroid of each binary image was calculated. The fluorescence image taken at ($\lambda_{\text{exc}} = 440$ nm, $\lambda_{\text{em}} = 540$ nm) was chosen as the reference. All other images were coregistered with the reference image in both x and y dimensions according to the positions of the centroids.

Two methods were used to process these fluorescence images, which were illustrated in Figs. 3(a) and 3(b). Figure 3(a) shows a method of discriminant analysis [28], which was implemented by the “classify” function in Matlab 7.2 (Mathworks, Inc. MA). The “quadratic” discriminant function was chosen for classification, in which multivariate normal densities with covariance estimated for each group are used in the analysis. The pathologist examined

the tissue to determine the exact location and boundary of tumors. To be safe, the pixels in a region smaller than the examined region were used for the training purpose. The training pixels in the coregistered images that respectively correspond to normal tissue, malignant tissue and the background were manually labeled using a graphical interface written in Matlab. The spectra associated with the training pixels were used to train the discriminant analysis classifier. Then the spectra for all pixels were fed into the trained classifier. A diagnostic image was generated as the result indicating the normal, malignant and background regions with different colors.



(a)



(b)

Fig. 3. Flowchart of (a) the discriminant analysis of spectral data; (b) the multi-variate discriminant analysis. In (b), ten most important principal component (PC) scores for full spectra analysis or all key PC scores for synchronous spectra analysis instead of original spectral data were used for classification. The left dotted rectangle indicates the training part while the right dotted rectangle indicates the classification part.

A multi-variate statistical method [29] shown in Fig. 3(b) offers an alternative way to reduce data dimension while maximizing the diagnostic capability of these fluorescence images. Principal component analysis (PCA) was first carried out to convert the fluorescence spectra associated with training pixels to uncorrelated principal components. The Wilcoxon rank

sum test was used to find principal components (PCs) that demonstrate most significant differences between normal and malignant regions. The key PC scores that explain most of the variance and demonstrate significant differences between normal and malignant pixels were used to train the same classifier as in the first method. Then the key PC scores of fluorescence spectra for all pixels were fed into the trained classifier to generate the diagnostic image. The procedure not only helps reduce data dimension but also removes redundant information in the raw data that may negatively affect the classification accuracy.

Both synchronous data and full spectra data were analyzed using the two methods. In the first method, totally 119 data points for each pixel were concatenated to form a single spectrum as the input in case of full spectra data. While in case of synchronous data, there were only 8-10 data points for each pixel as the input for classification. In the second method, the PCA of a single spectrum for full spectra data generated 119 PCs. Only the first 10 key PC scores were retained for classification in the analysis. And in case of synchronous data, all the PC scores that show significant differences between normal and malignant regions were retained for classification due to the small number of available data points. Because a pathologist can only tell roughly the locations of normal and malignant regions, the precise gold standard result for every pixel was obtained by classifying the pixels based on the full spectra data with the guidance of the pathologist. The specificity and sensitivity of classification using the synchronous data were calculated according to Eqs. (2) and (3). The classification accuracy is then the measure of the diagnostic potential of synchronous data relative to full spectra data.

$$\text{Specificity using synchronous data} = \frac{\text{Number of pixels classified as normal in both synchronous spectra analysis and full spectra analysis}}{\text{Number of pixels classified as normal in full spectra analysis}} \quad (2)$$

$$\text{Sensitivity using synchronous data} = \frac{\text{Number of pixels classified as malignant in both synchronous spectra analysis and full spectra analysis}}{\text{Number of pixels classified as malignant in full spectra analysis}} \quad (3)$$

3. Results

Figure 4 show the manually labeled training image overlaid on a representative raw fluorescence image. The normal region (blue polygon) exhibits higher fluorescence intensity while the malignant region (green polygon) shows lower fluorescence intensity. A circular field of view can be barely seen on the image.

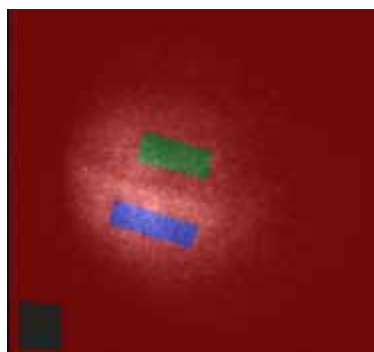


Fig. 4. The semi-transparent version of the manually labeled training image overlaid on a representative fluorescence image taken at ($\lambda_{\text{exc}} = 440 \text{ nm}$, $\lambda_{\text{emm}} = 540 \text{ nm}$). The blue region was manually assigned as normal, the green region was assigned as malignant and the dark gray region was assigned as the background.

Figure 5(a) show a diagnostic image overlaid on a representative raw fluorescence image, where the diagnostic image was generated by full spectra analysis with the first method [Fig. 3(a)]. Figure 5(b) show a diagnostic image overlaid on a representative synchronous fluorescence image, where the diagnostic image was generated by synchronous spectra analysis ($\Delta\lambda = 30$ nm) with the first method as well. The shape of the boundary between normal and background regions in Fig. 5(b) is similar to that in Fig. 5(a). The quantitative comparison was carried out by calculating the specificity and sensitivity of classification according to Eqs. (2) and (3). The results for a range of differential wavelengths ($\Delta\lambda = 30 - 90$ nm) are listed in Table 1. Three differential wavelengths, including 30 nm, 50 nm and 90 nm, yield best accuracies according to Table 1.

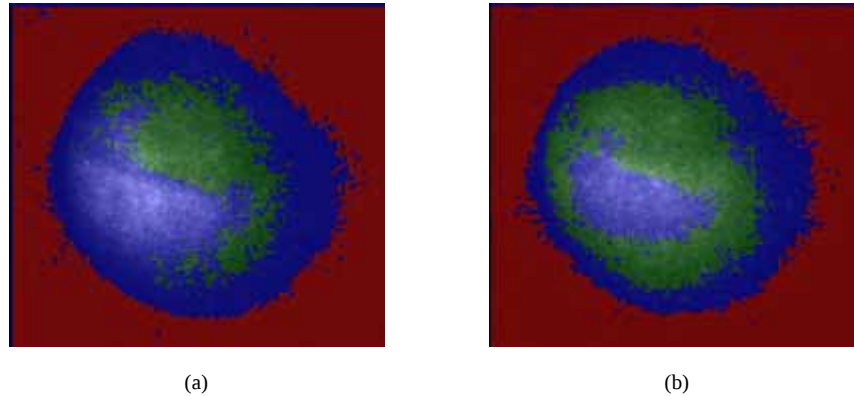


Fig. 5. The semi-transparent version of the diagnostic image generated (a) by full spectra analysis overlaid on top of a representative image taken at ($\lambda_{\text{exc}} = 440$ nm, $\lambda_{\text{em}} = 540$ nm) and (b) by synchronous spectra analysis ($\Delta\lambda = 30$ nm) overlaid on top of a representative image taken at ($\lambda_{\text{exc}} = 540$ nm, $\lambda_{\text{em}} = 570$ nm). The method of analysis was shown in Fig. 3(a). The central green region was classified as malignant and the surrounding blue region was classified as normal, while the red region was the background.

Table 1. Specificities and sensitivities of classification using synchronous data calculated according to Eqs. (2) and (3), in which the diagnosis image generated from full spectra analysis [Fig. 5(a)] was used as the gold standard. Both sets of data were analyzed with the first method [Fig. 3(a)].

$\Delta\lambda$ (nm)	Number of Points	Data	Specificity (%)	Sensitivity (%)
30	9	72	87	
40	10	37	96	
50	10	60	94	
60	9	25	99	
70	9	38	97	
80	8	25	97	
90	8	63	83	

Figure 6(a) shows the semi-transparent version of the diagnostic image generated by analyzing the images of the ten key PC scores, generated from full spectra analysis using the second method [Fig. 3(b)], overlaid on top of a representative fluorescence image taken at ($\lambda_{\text{exc}} = 440$ nm, $\lambda_{\text{em}} = 540$ nm). It should be noted that the ten key PC scores account for 99%

of the total variance contained in the raw data. Therefore a vast majority of information in the raw data has been taken advantage of. Theoretically this approach yields a more accurate diagnostic image than the raw data set because some fractions of the raw data that don't exhibit significant difference between malignant and normal regions have been removed.

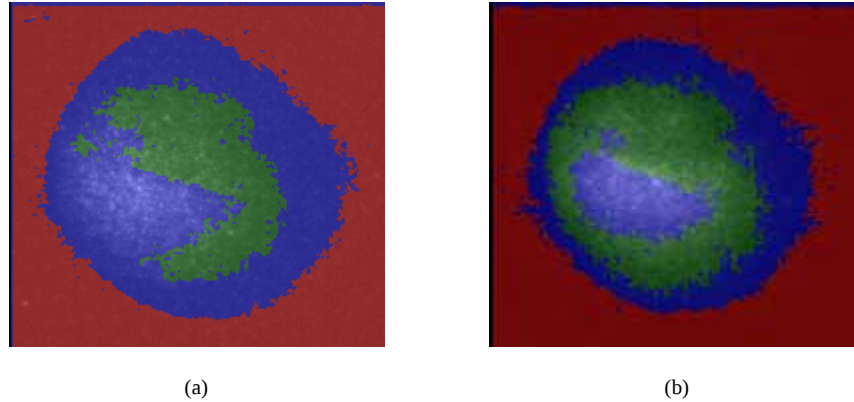


Fig. 6. The semi-transparent version of the diagnostic image generated (a) by analyzing the images of first ten key principal component (PC) scores calculated from full spectra data overlaid on top of a representative image taken at ($\lambda_{\text{exc}} = 440 \text{ nm}$, $\lambda_{\text{emmm}} = 540 \text{ nm}$) and (b) by analyzing the images of all key PC scores calculated from a single set of synchronous spectra data ($\Delta\lambda = 30 \text{ nm}$) overlaid on top of a representative image taken at ($\lambda_{\text{exc}} = 540 \text{ nm}$, $\lambda_{\text{emmm}} = 570 \text{ nm}$). The method of analysis was shown in Fig. 3(b). The central green region was classified as malignant and the blue region was classified as normal, while the red region was the background.

Synchronous images were also analyzed with the second method to ensure that the diagnostic potential of synchronous data was fully exploited and the generated diagnostic image for $\Delta\lambda = 30 \text{ nm}$ was shown in Fig. 6(b). Interestingly, Fig. 6(a) and Fig. 5(a) show considerable differences in the classification result especially in the bottom right corner of the malignant area (green region). This suggests that the use of those key PCs instead of full spectra did affect classification significantly. In contrast, there are no significant differences in the boundary between normal and malignant regions in Fig. 6(b) and Fig. 5(b), which infers that this set of synchronous data is close to the extracted PCs in terms of classification. The specificities and sensitivities of the diagnostic image generated from synchronous data were calculated according to Eqs. (2) and (3) again and listed in Table 2, for which the diagnostic image [shown in Fig. 6(a)] based on the first 10 sets of key PC scores generated from full spectra analysis was used as the gold standard. It can be seen from the second column that most PCs extracted from synchronous data demonstrated significant differences between normal and malignant regions. Three differential wavelengths, 30 nm, 50 nm and 90 nm, continue to yield best accuracies just as in Table 1.

Table 2. Specificities and sensitivities of classification using synchronous data calculated according to Eqs. (2) and (3), in which the diagnosis image generated from full spectra analysis [Fig. 6(a)] was used as the gold standard. Both sets of data were analyzed with the second method [Fig. 3(b)].

$\Delta\lambda$ (nm)	Numbers of Key PCs and All PCs	Specificity (%)	Sensitivity (%)
30	7/9	75	93
40	8/10	37	90
50	7/10	71	90
60	7/9	26	97
70	9/9	39	95
80	8/8	25	94
90	8/8	64	82

4. Discussion

We have shown that the classification results based on synchronous data analysis can achieve reasonably good accuracy compared to the results based on full spectra analysis when the differential wavelength is chosen appropriately. A great advantage of synchronous fluorescence imaging is that a much smaller number of data points are required while more than one fluorophores could still be detected. Furthermore, the contrast between fluorescence peaks and surrounding background in the spectrum can be significantly enhanced, from which the classification relied on the subtle spectral differences between malignant and normal tissues will benefit. This approach thus can dramatically reduce data acquisition time and still keep the classification accuracy reasonably high as seen in Tables 1 and 2.

In our measurements, synchronous imaging essentially scans the excitation and emission wavelength simultaneously while keeping the differential wavelength at a constant, which would be illustrated as a diagonal line across an EEM plot. Intuitively the choice of the differential wavelength should allow the synchronous scan to cross those important fluorescence peaks corresponding to known fluorophores. The optimization of the differential wavelength in a dilute mixture of multiple components has been discussed previously [30]. As inferred from that discussion, the optimal differential wavelength needs to be determined empirically given the complexity of tissue components. Tissues contain not only multiple fluorophores but also strong absorbers such as hemoglobin that could significantly alter the most valuable wavelengths for diagnosis. In practice, the differential wavelength can be varied from the smallest Stokes shift (difference between absorption and fluorescence peaks) to the largest Stokes shift of those most important endogenous tissue fluorophores, one or a few differential wavelengths that yield the best classification accuracy should be chosen as the optimal value(s).

Although the three differential wavelengths, 30, 50 and 90 nm, always demonstrate good accuracies in the above study, they are by no means unanimously the best differential wavelengths for tissue diagnosis. A few synchronous scans may be combined to achieve optimal classification. In addition to the discriminant analysis used in this study, some other classifiers have been reported to yield good performance, for example, support vector machine, artificial neural network and genetic algorithm. They can also be adapted to process spectral imaging data. In the future clinical study, these classifiers will be examined as well to achieve the balance between classification accuracy and data analysis time.

Given that it took 0.2 sec to acquire one image with this system, acquiring images for full spectra (totally 120 images) would theoretically take about 24 seconds. In contrast, acquiring images for synchronous spectra (totally 10 images) would take about 3 seconds, which is

much shorter than the time required to take images for full spectra. The whole process of data analysis to generate a single diagnostic image takes only a few seconds if the classifier is trained in advance. Therefore the synchronous imaging system can be used for clinical studies once all above procedures are automated, which is part of ongoing work.

5. Conclusion

We have developed a synchronous fluorescence imaging system to combine the great diagnostic potential of synchronous spectroscopy and the large field of view of imaging for cancer diagnosis. This system has been tested in a mouse skin model to capture synchronous fluorescence images. A simple discriminant analysis method and a more complicated multivariate statistical method have been developed to generate a single diagnostic image from a large number of raw fluorescence images. Moreover, it was quantitatively demonstrated that the diagnostic image generated from synchronous data is comparable to that generated from full spectral data in classification accuracy.

Acknowledgment

The authors would like to thank Dr. Stephen Kennel for providing the mice and cell lines used in this study. This work was supported by the National Institutes of Health under grant number RO1 CA88787-01.