Combination of Diffuse-reflectance and Fluorescence Imaging of Turbid Media

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ABSTRACT

We have used the cross-polarization method to eliminate the specular reflection and enhance the diffusive back-scattering of polarized fluorescence excitation light from the turbid media. The image of the ratio between fluorescence and cross-polarized reflection provides a map of the fluorescence yield, defined as the ratio of the autofluorescence emission to the illumination incidence, over the surface of the turbid media when excitation and collection of fluorescence are highly inhomogeneous. The simple ratio imaging technique shows the feasibility to detect early cancer, which usually starts from the superficial layer of tissue, based on the contrast in the fluorescence yield between lesion and normal tissue.

Keywords: Fluorescence, diffuse reflection, tissue, imaging

1. INTRODUCTION

Optical spectroscopy has been widely used in chemical analysis of biological samples. Since the mid-80s, the use of this technology for tissue classification has become a new approach in non-invasive medical diagnostics. In particular, the methods involved with laser-induced fluorescence (LIF) to characterize pathological states in human tissue have been extensively investigated^[1-7]. The tissues are usually classified based on the spectral characteristics of endogenous tissue fluorescence (autofluorescence). Careful *in vivo* studies on several organ sites with an optical fiber catheter revealed that the fluorescence yield of early lesions were almost always lower than that of the surrounding normal tissues, although the spectral lineshapes of early lesions and normal tissues vary both from individual by individual and within an individual^[4-7]. The algorithms based on the contrast in fluorescence yield between lesion and normal tissue can be used to discriminate early lesions. However, the point by point diagnosis by the use of an optical fiber catheter of fixed fluorescence excitation and collection geometry is not practical in clinical application. An imaging technique is desirable.

For examination of large area by an imaging device, recorded fluorescence power are strongly affected by the geometrical effects such as separation of the source-sample-detector, incident/emission angles and irregularity of sample surface. Without a mechanism to calibrate or compensate the geometrical effects, it is extremely difficult to compare the fluorescence yield at different sites in the imaged area, especially for *in vivo* measurements. Several groups have pursued different ways for mapping of fluorescence yield over the surface of turbid media. A non-imaging approach based on ratio of fluorescence intensity to reflection showed that the geometrical effects could be compensated, but the artifacts caused by the specular reflection of the tissue surface created much false positive^[2,3]. In ref. [6], an effort combining ultrasound and fluorescence spectroscopy showed that the accuracy of tissue characterization was improved by compensating the detector-sample separation, though the dependence of fluorescence intensity on the incident/emission angle was uncertain. A digital image processing method was reported to eliminate the geometrical effects in fluorescence imaging by taking the ratio of the fluorescence image to itself processed by the "moving average algorithm", a type of low pass filter^[7]. However, to ensure smoothing out the lesions and keeping the excitation and collection nonuniformities in the reference image, the algorithm requires that the lesion width is much smaller than the imaged area. Furthermore, if the spatial frequency of the low fluorescence areas caused by geometrical effects is overlapping with that of lesion, this low pass filter based image processing method will be unable to distinguish the lesion from the geometrical artifacts. In this study we introduce an imaging technique that allows for mapping of the fluorescence yield over the surface of a turbid medium by correcting the excitation and collection nonuniformities.

2. MATERIALS AND METHODS

The technique is based on a simple hypothesis. The reflection of the excitation light from the turbid media consists of three components: specular reflection, non-diffusive backscattering (such as single scattering) and diffusive backscattering. The diffusive components carry the information of excitation and collection geometry. Therefore, the geometrical effects of fluorescence imaging may be corrected by normalizing the fluorescence signal to the diffusive reflection recorded from the same site. Experimentally, a cross-polarization method can be used to reject the specular reflection and extract the diffusive components from the total reflection of excitation light. The tissue like sample (turbid medium) is illuminated with linearly polarized fluorescence excitation light and, by the use of a polarization analyzer, the cross-polarized image is recorded. Since forward scattering dominates the light propagation in the human tissue, the cross-polarized components in the reflection are mainly due to the multiple scattering and highly diffusive. The information carried in the cross polarization image are predominantly from beneath the superficial layer^[8,9]. The specular reflection and superficial information (mainly due to the single back-scattering), which cause the artifacts in correction of geometrical effects, are almost completely removed in the cross-polarized image. Taking the ratio of the fluorescence signal against the cross-polarized reflection recorded from the same position, a normalized fluorescence image is formed.

The experimental arrangement is shown in Figure 1. A laser with 200 mW output at wavelength of 457 nm delivered the excitation onto the tissue phantom through a linear polarizer and microlenses. The fluorescence and reflection signals from the sample were collected by a commercial endoscope and imaged to an 8-bit CCD camera. The angle between the optical axes of the endoscope and illumination optics was about 15°. Both the divergent angles of the illumination optics and collection angle of the endoscope were about 38°. The imaged area on the sample surface was about 12×12 mm at $\theta = 0^\circ$. The holder of a long-pass filter with cut-off wavelength at 470 nm and a polarizer with the polarization axis perpendicular to that of excitation light allowed for easily recording the fluorescence or cross-polarized images. The fluorescence and cross-polarized images were grabbed by a frame-grabber. To improve the signal to noise ratio, the fluorescence image was formed by taking average of 16 image frames.

Figure 1. Schematic diagram of the experimental setup used to collect the fluorescence and cross-polarized reflection images. F1: long pass filter; P1, P2: polarizers with polarization axis perpendicular to each other; θ : angle between the optical axis of endoscope and the normal of sample surface.

Tissue-simulating phantoms were made of gelatin with 20% solids dissolved in boiling deionized water, polystyrene spheres (0.55 μm in diameter), fluorescent dye mixture and dominantly absorbing blood with red cells hemolyzed. The contents of blood, which determined the absorption coefficients (μ_a) of tissue phantoms used in this study, were 2.5%, 5%, 7.5% and 10% by-volume, respectively. The concentrations of polystyrene spheres, the dominant scatterers in the phantoms, were set to 0.25%, 0.35% and 0.5% by-weight, respectively. The scattering coefficients (μ_s) and anisotropy factor (g) of tissue phantoms were calculated by Mie theory^[10,11]. In the wavelength region of 450 - 700 nm, μ_s for the concentrations of polystyrene microsphere at 0.25%, 0.35% and 5% were ranged from 95-39 cm^{-1} , 133-55 and 189-78 cm^{-1} , respectively. The g factor were ranged from 0.9-0.82. The composition of fluorescent dye mixture was carefully controlled to ensure its spectral lineshape similar to that of the autofluorescence of human tissue in the range of 450-700nm^[1,4,5]. The absorption coefficients of dye mixture in all tissue phantoms were kept at least a factor of 10 smaller than that of 2.5% blood, the lowest level of blood content in all phantoms. The absorption of dye mixture was negligible. This ensured the linearity between the fluorescence yield and the dye content in a phantom. The optical properties (μ_s , μ_a and g) of all phantoms were in the range of human tissue^[12].

3. RESULTS AND CONCLUSIONS

In the first experiment, we demonstrate that the excitation and collection nonuniformities of fluorescence imaging of the homogeneous tissue phantom with flat surface can be corrected by the ratio imaging method. The optical properties of six phantoms were set by the combination of three blood contents (2.5%, 5%, and 7.5%) and two microsphere concentrations (0.25% and 0.5%). In practice, the geometry of imaging was changed by rotating the sample along the axis across the point-A and perpendicular to the plane of the page in Figure 1. The fluorescence and cross-polarized images were collected from all tissue phantoms at $\theta = 0^\circ$ – 60° with increment of 15° . For a fair comparison, the mean gray levels of all the fluorescence images and ratio images of fluorescence against cross-polarized reflection have been adjusted to 128, half of the full gray levels of an 8-bit image. The typical fluorescence and the ratio image are shown in Figure 2. The histograms of the gray level in the figure indicate the homogeneity of image. As can be seen in Figure 2, the gray level of fluorescence image varies in wide range, while the gray level of ratio image has a very narrow distribution around 128, the mean gray level. It was found that standard deviation (S.D.) of gray level for the fluorescence images recorded from all six tissue phantoms varied from 24 to 39 at $\theta = 0^\circ$ – 60° . In contrast, S.D. of all ratio images was below 3 and not sensitive to θ and optical properties of samples. This indicates that the geometrical effects have been corrected to a great extent.

Figure 2. Images collected from a sample with blood content of 2.5% and 0.5% microsphere at $\theta = 30^\circ$.

The second experiment was designed to evaluate the performance of the ratio imaging technique on inhomogeneous tissue-like turbid media. To build an inhomogeneous tissue phantom, parts of homogeneous sample were replaced with the sample of different optical properties or fluorescence yield. The structures of three representative inhomogeneous samples are shown in Figure 3. The simulating lesions of fluorescence contrast phantom were made with the sample of the same optical properties and fluorescence yield about 82% of surrounding sample. Two holes of depth ~ 2.5 mm were made to represent the irregularity of the sample surface and create interference for identification of simulating lesions. The absorption contrast phantom was made with samples of different μ_a and the same μ_s . The scattering contrast phantom was made with samples of different μ_s and same μ_a . The fluorescence dye content was uniform in both phantoms. The raw fluorescence and ratio images of fluorescence contrast tissue phantoms are displayed in Figure 4. Apparently, one can not distinguish the simulating lesions from the holes in the raw fluorescence image of fluorescence contrast phantom. In contrast, only the simulating lesions appear clearly, and the difference in fluorescence yield between simulation lesion and normal tissues were recovered. To explore the possibility to simplify the technology by using the similar approaches reported in ref. [2,3], we took the ratio of fluorescence to direct reflection. The direct reflection and ratio images shown in Figure 4 demonstrate that the false signals caused by the specular reflection create severe interference to identify the signal of simulating lesions. Comparing the raw and ratio images of absorption contrast phantom in Figure 5, it was found that the change of fluorescence signal caused by difference of blood content was corrected. This indicates that the effect of absorption variation on diffusive reflection and fluorescence may cancel each other. We observed that the variation of scattering property in scattering contrast phantom has not been corrected in the ratio image. This is because the cross-polarized reflection is much more sensitive to the scattering properties than fluorescence as shown in Figure 5. The variation of tissue scattering property may introduce artifacts to the ratio imaging technique. However, the scattering coefficient is mainly

determined by the tissue structure. The scattering difference between the premalignant early lesion and normal tissue may not be significant due to their histological similarity.

Figure 3. Structures and compositions of inhomogeneous phantoms. Left: Fluorescence contrast, Blood content: 5%, Concentration of microsphere: 0.35%; Right: For absorption contrast, Microsphere: 5%; Blood: 10% in area A, 2.5% in area B and 5% in surroundings. For scattering contrast, Blood: 5%; Microsphere: 0.5% in area A, 0.35% in area B and 0.25% in the surroundings.

(a)

(b)

Figure 4. Experimental results for fluorescence contrast phantom: (a) Raw fluorescence image and line profile analysis, (b) Ratio image and line profile analysis, (c) Direct reflection and ratio images.

Figure 5. Raw fluorescence, cross-polarized reflection and ratio images recorded from absorption contrast phantom (above) and scattering contrast phantom (below).

It should be pointed out that most of human tissues have multiply layered structure and the distribution of endogenous fluorophores as the function of depth. The evaluation of the ratio imaging method on the real tissue may be more complicated. Our future work will be focused to study the *in vivo* examination on various tissues by using a specially designed endoscopic system based on the concepts reported in this paper. The effect of possible variation in tissue structure/scattering on the mapping of fluorescence yield will also be investigated.

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4. REFERENCES

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