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Contrast Enhancement for *in vivo* Visible Reflectance Imaging of Tissue Oxygenation

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Abstract

Results are presented illustrating a straightforward algorithm to be used for real time monitoring of oxygenation levels in blood cells and tissue based on the visible spectrum of hemoglobin. Absorbance images obtained from the visible reflection of white light through separate red and blue bandpass filters recorded by monochrome CCDs are combined to create enhanced images that suggest a quantitative correlation to the degree of oxygenated and deoxygenated hemoglobin in red blood cells. The filter bandpass regions are chosen specifically to mimic the color response of commercial 3-CCD cameras, representative of detectors with which the operating room laparoscopic tower systems are equipped. Adaptation of this filter approach is demonstrated for laparoscopic donor nephrectomies in which images are analyzed in terms of real time, *in vivo* monitoring of tissue oxygenation.

Introduction

A growing need exists for new instrumental techniques to aid physicians in quantitatively assessing organ and tissue dysfunction in real time. Adaptation of physiological biomarkers for this purpose, such as blood and tissue oxygenation measurements, provide wide ranging applications, for example, in cancer diagnosis^{1,2}, in monitoring tissue viability for organ transplants³, and in determining the severity of a stroke⁴. That is, decreases in blood supply can result in ischemic injury, which, in turn may lead to hypoxia, a condition that arises when the lack of oxygen in the tissue disrupts metabolic processes. In the case of organ transplantation, viability depends in part on the capacity of the organ to undergo reperfusion and reoxygenation. Since current *in vivo* techniques for monitoring organ viability, specifically in kidney transplants, include primarily urine output and the visual observation of the surgeon, a requirement arises for accurate, real time observation of tissue oxygenation during surgery.

Noninvasive spectroscopic methods offer tremendous promise for tissue perfusion and oxygenation measurements since they are inherently highly sensitive and quite specific. Resonance Raman spectroscopy has demonstrated an ability to monitor, *in vivo*, changes in tissue oxygenation based on changes in heme associated spectral features.^{5,6} Hemoglobin (Hb), which comprises 80-90% of the protein found in red blood cells (erythrocytes), exhibits well established spectroscopic characteristics in both the oxygenated and deoxygenated states.⁷ Near infrared spectroscopy has also been used to monitor tissue oxygenation based on changes in the absorbance spectra of oxy- and deoxyhemoglobin.^{8,9} Other spectroscopic methods that have been used to monitor tissue oxygenation include the use of a non-contact laser tissue blood flowmeter¹⁰, visible light spectroscopy^{1,11,12}, and laser autofluorescence imaging^{13,14}. In autofluorescence imaging, a 335nm laser excites the fluorescence from NADH, which is believed to accumulate in tissue during ischemia.¹⁵ None of these techniques,

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however, provide chemically specific information, large field of view imaging, and *in vivo* detection simultaneously.

Laparoscopic methods, unlike open surgeries, require special considerations that can be problematic as a consequence of incorporating additional instruments and probes. Laparoscopic surgery is a minimally invasive operating technique, resulting in shorter hospital stays, faster return to work, and a decreased risk of infection when compared to open surgery^{16,17}; when available, patients often choose laparoscopic surgery over the more traditional open surgery. The introduction of specialized instrumentation into an operating room, however, often inhibits wide acceptance of new methodologies. We propose further adapting the laparoscopic visible reflectance method of *in vivo* applications, particularly since a light source already exists for illuminating the internal organs of the patient and no additional operating room equipment is required. Specifically, existing laparoscopic towers already incorporate the requisite light source, and light delivery and collection optics, as well as wavelength filters and a detector (namely, the three charge-coupled device camera, or, 3-CCD camera).

A 3-CCD camera uses red, blue, and green bandpass filters in front of three separate monochrome CCDs to record and reconstruct a color image. Manufacturers of laparoscopic towers have now generally replaced the single charge-coupled device (1-CCD) camera in operating rooms with 3-CCD cameras due to the 3-CCD's increased color sensitivity and extended color palette range. The 3-CCD camera maintains spectral information, although extensively binned, of the object being viewed. The advantage is that the intensity of reflected light can be monitored separately for the red, green, and blue channels for resolving the contributions of that which appears to the eye (or common camera), as a composite color such as orange or yellow.¹¹ In this report, we present proof-of-concept experiments to illustrate the manner in which filtered images can be straight forwardly combined to create a contrast enhanced image that is specifically sensitive to changes in blood oxygenation. This technique is first simulated and validated using oxygenated and deoxygenated blood samples. The contrast enhanced images are then adapted to existing 3-CCD cameras in operating room laparoscopic towers to provide real-time, *in vivo* tissue oxygenation information, an invaluable tool for assisting laparoscopic surgeons.

Experimental

Oxygenated blood was collected in a glass vial after pricking a finger with a sterile needle. Deoxygenated blood samples were prepared by the addition of sodium dithionite (85% Na₂S₂O₄, Sigma-Aldrich, St. Louis, MO) to oxygenated blood. UV-visible spectra were acquired of all samples to verify the oxygenation state of the hemoglobin. Spectra were collected at 1 nm intervals using a Cary-100 Bio UV-Vis spectrometer (Varian, Palo Alto, CA). Blood samples were diluted to a concentration of 0.1% blood in phosphate buffered saline solution to minimize the amount of blood needed and to reduce sample absorbance to a level within the dynamic range of the spectrometer.

Erythrocyte images were acquired using an upright Olympus BH-2 microscope equipped with an Olympus MDPlan 80×/0.90 NA objective and a Nuance™ multispectral imaging system (CRI, Inc., Woburn, MA) optimized for fluorescence spectroscopy. Briefly, the Nuance™ consists of a single color CCD camera and a liquid crystal tunable filter (LCTF) with a 30 nm full-width at half-maximum (FWHM) bandpass around the tuned color frequency. Blood samples were spotted onto a clean glass microscope slide and covered with a glass coverslip to minimize pathlength and to provide a consistent sample thickness. Hydration was preserved; the samples were imaged immediately to minimize cellular reuptake of oxygen. Cells were examined at high magnification to correlate color changes in the individual cells with

oxygenation, as determined by the UV-visible spectroscopy of the whole blood samples. To mimic the operating room's commercial 3-CCD camera, red blood cell images were taken at 460, 520, and 590 nm, with 84 ms acquisition times, using the Nuance™ multispectral imaging system. Identical exposure times were used for both the oxygenated and deoxygenated samples, allowing meaningful comparisons at each wavelength. For the red blood cell analysis and comparison, at least seven blood cells were examined for both the oxygenated and deoxygenated blood. The mean relative intensity was calculated for each cell from a 20 pixel $\times$ 20 pixel region of interest (ROI).

Absorbance images of erythrocytes are created from referencing a background transmittance signal (T_0), to the transmittance signal (T) in the presence of the sample. The absorbance (A) is then the relationship shown in equation 1:

$$A = -\log(T/T_0) \quad \text{Eq. 1}$$

The derived absorbance value represents the change in light intensity resulting from the interaction of light with the chromophore in the sample. For opaque samples, such as tissue, transmittance is not possible. In the case of such samples, however, reflectance measurements have been demonstrated to provide an apparent absorbance that is qualitatively the same as that derived from transmission samples.^{12,18,19} The actual absorbance obtained from hemoglobin in the translucent sample of erythrocytes is used to infer information about oxygenation from the apparent absorbance of hemoglobin in tissue.

Surgical images were obtained using a Storz laparoscopic tower (Tuttlingen, Germany) equipped with a 3-CCD camera attached to the laparoscope. The Storz laparoscopic tower consists of an insufflator, a 300 W xenon lamp (fiber-coupled to a 30° viewing angle laparoscope), the detector (in this case, a 3-CCD camera) and a digital video recorder. Laparoscopic images were converted to absorbance images using the reflection from white gauze, commonly available in operating rooms, as the 100% reflection and white correction standard. Uncompressed TIFF (tagged image file format) images were extracted from individual frames of the surgical video and scaled from 0-255. Enhanced images were prepared by separating the filtered responses (RGB) and subtracting the blue CCD channel absorbance from the red CCD channel absorbance. The enhancement images are plotted using a red-blue color map, where red corresponds to small differences between the red and blue filtered CCD channels and blue corresponds to large differences between the red and blue filtered CCD channels.

Results and Discussion

The UV-visible spectra obtained from oxygenated and deoxygenated blood are displayed in Figure 1. The oxygenated blood shows characteristic absorption peaks at 416, 541, and 577 nm (solid black curve). The deoxygenated blood displays absorbance peaks at 430 and 556 nm (gray curve). These absorption bands arise from the π to π^* transitions of the B and Q bands associated with Hb in oxygenated and deoxygenated blood.⁷ Sodium dithionite, used to deoxygenate blood samples, contributes an absorbance towards the UV region of the visible spectrum in deoxygenated samples.²⁰ To permit accurate comparison of extinction coefficients, the Hb absorption curves were normalized for pathlength and concentration, and the sodium dithionite spectral contribution was subtracted. Gaussian curves are overlaid onto the Hb spectra in Figure 1 to simulate the transmission of the LCTF at the red (590 nm), green (520 nm), and blue (460 nm) wavelengths. The Gaussian curves are plotted with an arbitrary intensity, but with a FWHM corresponding to the bandpass of the LCTF. It is clear from Figure

1 that the deoxygenated blood exhibits a greater absorption than oxygenated blood in the blue channel, as the absorption maximum of the B band π to π^* transition moves from 416 nm to 430 nm (towards the transmission maximum of the blue bandpass filter).

The 3-CCD enhancement arises directly from the absorption properties of Hb. In Figure 1, the total absorbance (integrated area under the absorbance curve) is nearly identical for deoxygenated and oxygenated Hb in the region associated with the red channel. However, when blood is deoxygenated, the shift in the B band results in a greater absorbance in the blue channel. The differences between the red and blue channel absorbances, ΔS , create a new metric for blood oxygenation. This new metric scales linearly ($R^2 = 0.99996$) with varying components of oxygenated and deoxygenated blood, as shown in Figure 2.

This contrast enhancement can be demonstrated mathematically. The absorbance response from each color channel can be expressed as shown in equation 2,

$$S_{\lambda} \cong \int_a^b f(\lambda) A(\lambda) d\lambda, \quad \text{Eq. 2}$$

where the absorbance signal (S) over a particular wavelength range (λ) is evaluated as an integral of the measured absorbance [$A(\lambda)$] over the wavelength range (a to b) convoluted with the filter attenuation [$f(\lambda)$] over the same wavelength range. The filter attenuation is modeled as a normal distribution around the transmission maximum with a half width at half maximum (σ) corresponding to the bandpass of the filter. The contrast between two different filters is then simply the difference in the two integrals as shown in equation 3,

$$\Delta S_{\text{Red-Blue}} = S_{\text{Red}} - S_{\text{Blue}}. \quad \text{Eq. 3}$$

For the purposes of our experiment, the limits of the integral are set as $\pm 3\sigma$ (blue: 415-505 nm, red: 545-635 nm), to account for >99% of the measured absorbance. From this mathematical model, the calculated integrals for deoxygenated and oxygenated Hb are nearly identical (within 2% of each other) over the wavelength range of the red channel; however, significant differences are obvious in the blue channel for which deoxygenated Hb exhibits 20% more absorbance relative to oxygenated Hb. These differences in the blue channel provide the inherent contrast for our method. However, standardization of the differences in the red channel provides a reference for correlating the changes in hemoglobin oxygenation in contrast to volume changes or optical effects, such as glare or shadows, that may cause intensity variations when using a single channel detector in a laparoscopic approach.

To validate the 3-CCD enhancement, we examine red-green-blue (RGB) images at 800 $\times$ magnification of the oxygenated and the deoxygenated red blood cells, as shown in Figures 3A and 3B, respectively. Some of the deoxygenated cells exhibit a morphological change, appearing shriveled in shape. This contraction is due to the formation of Heinz bodies, due to hemolysis caused by oxidative injury; in this case, the oxidative stress has been induced by the addition of sodium dithionite.²¹ The shriveled cells were excluded from calculations; only cells exhibiting normal morphology were included in the data analysis. Note, that in excluding the Heinz bodies the RGB images show only subtle changes in erythrocyte physical appearance. Displayed in Figure 4 are the gray scale absorbance images (plotted in false color) of the same oxygenated and deoxygenated erythrocytes shown in Figure 3 that were obtained at the three approximated filter wavelengths, as collected with the Nuance™ imaging system, namely 590 nm (red), 520 nm (green), and 460 nm (blue). Bright colors indicate the strongest absorption

of light; black indicates the weakest absorption of light. At best, the differences in the single color images of oxygenated (left) and deoxygenated (right) blood are not dramatic and are difficult to distinguish.

The subtle differences between the images in Figure 4, however, can be enhanced by applying the algorithm in Eq. 2 to the individual CCD responses. Based on the differences observed in the UV-visible spectra, spectroscopically enhanced images of the oxygenated red blood cells (Figure 5A) and of the deoxygenated red blood cells (Figure 5B) were constructed by subtracting the blue CCD response from the red CCD response. Differences between the oxygenated and deoxygenated cells are now readily visible; in particular, the center of the deoxygenated cell is clearly darker than the oxygenated cell, indicating that the red blood cell exposed to sodium dithionite is, in fact, less oxygenated than the untreated cells.

The examination of at least seven red blood cells for each blood sample indicates a clear trend. Figure 6 shows a bar graph demonstrating enhancement image differences observed in the heme-containing center of the blood cells. The mean intensity value of the oxygenated cells is consistently greater than the mean intensity value of the deoxygenated cells. This implies that a threshold value could be established between mean intensity values of 133 ± 5 and 122 ± 4 (mean $\pm$ standard deviation) for the oxygenated and deoxygenated cells, respectively. A two-tail student's t-test yields a p -value of 0.0005, clearly indicating the mean values exhibit statistically significant differences. The variance in the values between cells may be associated with nonuniformities in the distribution of the light illumination throughout the image. For purposes of comparison in Figure 6, every effort was made to compare red blood cells from similar locations and illumination conditions within the field of view of the image. Despite this variance, statistically significant differences are observed.

In principle, the same information presented above could be obtained without the LCTF, but rather by placing three different bandpass filters in front of the camera and acquiring an image for each bandpass filter. This is slightly problematic for small specimens, like blood cells, where diffusion and movement between images creates alignment issues; in this case, the LCTF has the advantage of being tuned rapidly, allowing for faster acquisition times. For large samples, however, where position is maintained easily, the use of colored bandpass filters is a cost effective alternative. In the case of the 3-CCD camera, both approaches are circumvented by using three bandpass filters and three CCDs, enabling simultaneous image capture.

We now apply the actual 3-CCD enhancement procedure to video footage of a single case of laparoscopic donor nephrectomy (kidney removal for transplantation), where thrombotic injury was reported by the surgeon. Figure 7A displays the baseline kidney image, as observed by the surgeon; the kidney has been outlined in a yellow dashed line for clarity. Note that in this image, the kidney appears to have a relatively uniform pink coloration, indicating that the kidney is perfused and oxygenated. The 3-CCD enhancement is overlaid onto the original image (Figure 7B) enabling visual registration for the surgeon. Three ROIs, delineated by the white boxes, provide a baseline relative intensity value (0.835 ± 0.040). In Figure 7C, however, the kidney has sustained thrombotic injury (vascular obstruction) in which blood flow to the kidney was reduced. It is clear by the intensity of the blue coloring in Figure 7D that the lower left portion of the kidney suffers from reduced tissue oxygenation. The relative intensity value of the ischemic portion of the kidney is 0.695 ± 0.051 . Using the student's t-test sample means (two-tailed) comparison, the mean relative intensity value for the ischemic portion of the kidney is significantly decreased from the mean baseline relative intensity value ($p=0.020$). Thus, adaptation of the currently used 3-CCD camera offers definite promise for aiding the laparoscopic surgeon in detecting loss of tissue oxygenation in real-time during surgery without the need for additional operating room equipment.

Conclusion

Controlled experiments using blood cells illustrate that the filtered response generated by an operating room's existing 3-CCD camera provides a statistically significant measurement for discerning the relative oxygenation of hemoglobin in a biological system. Tissue oxygenation is linked to numerous conditions, such as ischemic injury in kidney transplants, tumors, and thrombosis, which benefit from real-time *in vivo* diagnostics. This image enhancement method is easily implemented in currently outfitted operating rooms, as laparoscopic towers are already equipped with the necessary instrumentation for creating real time, contrast enhanced visualizations.

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Figure 1. Absorption spectra of oxygenated (solid black) and deoxygenated (solid gray) blood are shown normalized for pathlength and concentration. Gaussian curves are overlaid to illustrate the transmission of the three filter regions measured and used to model the 3-CCD camera.

Figure 2.

The linear dependence of the $\Delta S_{\text{red-blue}}$ response, simulated from the absorption data, is plotted for varying mixtures of oxygenated and deoxygenated Hb. The data points are calculated from the absorbance curves in Figure 1 with a line fit to these points.

Figure 3.
The gray scale RGB images of oxygenated erythrocytes (A) and deoxygenated erythrocytes (B) are shown at 800 $\times$ magnification.

Figure 4.

The blue (A & B), green (C & D), and red (E & F) filtered absorbance images are shown of oxygenated blood (left) and deoxygenated blood (right). Bright colors indicate greater absorption and black indicates no absorption.

Figure 5.
Enhanced images are shown of an oxygenated (A) and deoxygenated (B) erythrocyte.

Figure 6.

The graph shows measured $\Delta S_{\text{red-blue}}$ values obtained from the center of different oxygenated and deoxygenated erythrocytes. The error bars represent the standard deviation of the pixel values within each ROI. A dashed line is drawn to illustrate a possible threshold value between the two states.

Figure 7.

Displayed are images acquired with the 3-CCD camera in a laparoscopic tower during a donor nephrectomy before (A) and after (C) a thrombosis in the kidney. The yellow dashed line was added to guide the eye to the kidney in the visible images (A & C). A relatively uniform level of oxygenation is indicated in the 3-CCD enhanced image of the kidney before the thrombosis (B). Significant tissue deoxygenation is perceived by the intense blue color seen in the left portion of the kidney after the thrombosis has occurred (D).