Visible Reflectance Hyperspectral Imaging: Characterization of a Noninvasive, in Vivo System for Determining Tissue Perfusion

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We characterize a visible reflectance hyperspectral imaging system for noninvasive, in vivo, quantitative analysis of human tissue in a clinical environment. The subject area is illuminated with a quartz–tungsten–halogen light source, and the reflected light is spectrally discriminated by a liquid crystal tunable filter (LCTF) and imaged onto a silicon charge-coupled device detector. The LCTF is continuously tunable within its useful visible spectral range (525–725 nm) with an average spectral full width at half-height bandwidth of 0.38 nm and an average transmittance of 10.0%. A standard resolution target placed 5.5 ft from the system results in a field of view with a 17-cm diameter and an optimal spatial resolution of 0.45 mm. The measured reflectance spectra are compared.8 Later, measurements of the oxygen saturation of hemoglobin were refined by comparing the optical densities of blood at 660 nm and at a reference wavelength, 805 nm, the isobestic point for oxy- and deoxyhemoglobin.9 Additional approaches developed over the years resulted in a clinical oximeter clipped to a patient’s fingertip.10–11

Although oximetry represents a commonly used technique for determining spectrometric changes in hemoglobin characteristics, the method only measures oxygen saturation at either one or several sample points, which dramatically limits the technique to an assessment of a small region of tissue. Recent oximetry studies using specifically the near-infrared (NIR) spectral region, however, have been used to image tissue perfusion.12–14 Since the NIR spectral interval is complicated by the presence of both overlapping water bands and vibrational overtones, oximetry methodologies employing visible reflectance data provide a more straightforward manner in, for example, the determination of tissue perfusion and reperfusion as related to clinical events. In addition, oxygen saturation measurements in the clinical environment using visible reflectance imaging, as emphasized in this report, have the distinct advantage of visualizing the capillary circulation processes where oxygen is exchanged, which in this case is within a few millimeters of the skin’s surface. In contrast, NIR spectroscopic and other analytically based phlebotomy techniques typically examine blood characteristics within the deeper and larger blood vessels from which only inferences regarding oxygen exchange are determined.

Since the clinical usage of visible reflectance hyperspectral imaging is nonexistent at the present, we developed a technique to collect spectroscopic data from a large array detector at the detection of oxy- (HbO₂) and deoxyhemoglobin (deoxy-Hb) through the skin by a device consisting of a lamp, filter assembly, and photocell led to the original ear oximeter in which short- and long-wavelength light were passed through the tissue and compared.8 Later, measurements of the oxygen saturation of hemoglobin were refined by comparing the optical densities of blood at 660 nm and at a reference wavelength, 805 nm, the isobestic point for oxy- and deoxyhemoglobin.9 Additional approaches developed over the years resulted in a clinical oximeter clipped to a patient’s fingertip.10–11

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multiple, contiguous wavelengths at narrow spectral bandwidths. A critical component of our novel imaging instrumentation involves recent advances in liquid crystal tunable filter (LCTF) devices, which are representative of compact, electronically tunable, spectral notch filters with imaging capabilities. LCTF systems consist specifically of stacked stages containing a linear polarizer, a birefringent element, and a liquid crystal wave plate.\textsuperscript{15–20} The advantage of an imaging system utilizing an array detector is that each detector pixel allows a determination of a molecular spectrum from which the characteristic state of the tissue is assessed. In the present discussion, we demonstrate the utilization of this noninvasive imaging system by analyzing a quantitative model of vascular occlusion and subsequent perfusion as a function of time.

**EXPERIMENTAL SECTION**

**System Description.** The visible reflectance hyperspectral imaging system employs a broadband visible source and optics for illumination, an LCTF for spectral image filtering, and a charge-coupled device (CCD) detector for image collection. Data are acquired at numerous wavelengths (typically >100) and at tens to hundreds of thousands of spatial locations for defining a hyperspectral image cube.

Figure 1A displays the configuration of the reflectance imaging system, which consists of a source, optics, tunable filter, and detector. A 100-W quartz–tungsten–halogen (QTH) broadband source produces a stable output of spectral radiation spanning the ultraviolet (UV), visible, and NIR regions. The lamp is housed in a protective enclosure containing two condensing lenses and two rear reflector units (Oriel Instruments, Stratford, CT), allowing simultaneous illumination of the sample from two independent directions to minimize shadowing. The QTH source is powered by a radiometric power supply (Oriel Instruments, Stratford, CT), which maintains a constant current for long-term stable illumination with minimal light ripple (<0.05%rms). A long-pass UV filter with <5% transmission below 395 nm (Oriel Instruments) and a short-pass NIR filter with <5% transmission above 835 nm (Oriel Instruments) are used initially to filter the light. Subsequently, the radiation is focused into UV–visible–optimized liquid light guides (LLGs) (Oriel Instruments) whose large-diameter cores (8 mm) allow for a high light throughput in the visible spectral interval, while filtering the NIR spectral range (<15% transmission above 740 nm) restricting the radiation to the visible region of the spectrum, allowing a subject’s skin, for example, to be illuminated either with an increased intensity or for a longer duration, while still minimizing the potential of light-induced damage to the tissue. We note that data on the visible reflectance spectra of HbO\(_2\) and deoxy-Hb from skin tissue indicate that the radiation, which is re-emitted from the dermal layer, penetrates typically 1–2 mm below the skin surface.\textsuperscript{21} Beam-shaping optics direct the excitation from the LLGs to the subject and allow a circularly illuminated area to be varied anywhere from a 2- to 20-cm diameter. A front surface mirror (Newport, Irvine, CA) placed at a 45° angle steers the light reflected from the subject to the high-resolution LCTF (Cambridge Research & Instrumentation, Inc.).

![Figure 1: In vivo visible reflectance hyperspectral imaging system.](image-url)

(A) The instrumentation consists of a quartz–tungsten–halogen light source (1) that illuminates the subject with light filtered to remove UV and IR radiation (2) and is transferred by liquid light guides (3) to the subject via the beam-shaping optics (4). The diffuse reflectance is guided by a front surface mirror (5) to an LCTF (6) and a lens (7), which focuses an image onto the CCD detector (8). Data are digitized and archived on a computer attached via the cables (9). (B) A reflectance image of a portion of a USAF 1951 resolution target (group 2 element 3, and group 1 elements 3 and 4) is used to generate a contrast transfer function analysis. A line pair consists of one white and one dark bar. A line pair from the region labeled 3, on the left, corresponds to 3.175 mm and has a spatial frequency of 0.315 line pairs/mm, while the pairs labeled 3 on the right correspond to 1.587 mm with a spatial frequency of 0.630 line pairs/mm. (C) A row of pixels taken along the horizontal dotted line in (B) is plotted. The maximum and minimum intensity for each set of bars (spatial frequency) is used to determine the percent contrast. In this example, it can be seen that as the spatial frequency of line pairs increases, the percent contrast decreases.

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prepared by standard methods$^{24}$ using blood collected from a healthy, nonsmoking individual. The erythrocytes were washed with isotonic saline and centrifuged at 10 000 rpm for 10 min. The 100%HbO$_2$ sample was prepared by exposing the hemolysate to oxygen. Seven milliliters of hemolysate was mixed with 30 mg of sodium dithionite (Sigma, St. Louis, MO) to yield the 100%deoxy-Hb sample. The resulting reference spectra were measured using the visible reflectance hyperspectral imaging system, with characteristic peaks at 541 and 576 nm for HbO$_2$ and a single peak at 555 nm for pure deoxy-Hb.$^{5,6}$

**Data Acquisition.** The subject was seated in a comfortable position in order to minimize movement of the area of the skin to be imaged, which in this case is the palmar region of the hand. A baseline, hyperspectral image data cube was acquired with the subject at rest, followed by occlusion by a blood pressure cuff and four successive reperfusion measurements with data being acquired at each phase. The experimental timeline, depicted in Figure 2, shows the time progression of the experimental events and data acquisition; the baseline measurement is acquired 2 min prior to the onset of the induced ischemia. The occlusion measurement, modeling acute ischemia, was acquired 2 min after pressure was applied using a hand aneroid sphygmomanometer (Welch Allyn, Arden, NC). The first reperfusion measurement, which monitors reactive hyperemia, was acquired immediately after releasing the cuff pressure. The subsequent reperfusion measurements were acquired 6, 8, and 10 min postocclusion.

Each hyperspectral image cube consists of 121 individual images, collected from 525 to 645 nm in 1-nm increments. This interval spans the spectroscopically relevant regions of HbO$_2$ and deoxy-Hb. The measured spectra were filtered using a Savitsky–Golay smoothing filter for a final spectral range of 533–631 nm. The full CCD chip (768 × 512 pixels) was binned by 3 × 3 pixels for a final image size of 256 × 170 pixels; this increases sensitivity and allows the experiment to be completed in a time consistent with that afforded in a clinical setting. The exposure time for each image was 250 ms, resulting in an entire reflectance hyperspectral image cube being acquired and saved to disk in ~2 min. A background image cube of a 99.9% reflectance standard (Spectralon, LabSphere, North Sutton, NH), acquired under the same conditions as the subject image cube, was employed to minimize instrument response and to convert from measured reflectance to apparent absorbance.

**Data Analysis.** The determining the percentage of HbO$_2$ is a two-step process. First, the measured visible reflectance spectra are quantified in terms of apparent absorbance, A$_{xy}$(λ)$_i$,$^{3,6,25}$ a ratio between reflected sample radiation, R$_{xy}$(λ)$_i$, and the reflected radiation from a certified 99.9% reflectance standard,$^{26}$ R$_{xy}$(λ)$_o$, measured at wavelengths λ$_i$ for the spatial coordinates x and y, as shown in eq 1.

\[
A_{xy}(\lambda_i) = \frac{\log(R_{xy}(\lambda_i) / R_{xy}(\lambda_o))}{100}
\]  

In the second step, the apparent absorbance spectra, a convolution of the HbO$_2$ and deoxy-Hb, are transformed to the

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percentage of HbO2 by deconvoluting the measured spectra into two intrinsic components. Specifically, a measured spectrum is deconvoluted by performing a least-squares fit for each detector pixel to determine the best linear combination of the HbO2 and deoxy-Hb reference spectra. The multivariate regression of the spectrum at pixel position (x, y) is given by

\[ C_{kj} = S_{kj} R_j + e_{kj}, \] (3)

where \( C \) is a linear, multidimensional, deconvoluted contribution matrix composed of \( k \) pure components, in this case, HbO2 and deoxy-Hb, and \( j \) measured samples corresponding to each detector pixel \((x, y)\). The unknown sample data, \( R \), are functions of wavelength, \( \lambda_i \), and position, \( j \). The sensitivity matrix, \( S \), is given by

\[ S_{kj} = (P^t_{k\lambda} P_{k\lambda})^{-1} P^t_{k\lambda}, \] (4)

where \( P \) is the matrix of pure component spectra and \( t \) represents the transpose operation. Finally, \( e_i \) is a matrix of residuals associated with the regression. Using the pure component reference spectra of fully oxygenated hemoglobin (100% HbO2) and completely desaturated hemoglobin (100% deoxy-Hb) and substituting eq 3 into eq 2, one establishes a determination of the percentage of HbO2 at each sampling area. For improved accuracy, the deconvolution methodology was calibrated by assuming a linear relationship, as in previous studies, between the known value (100% HbO2 for pure oxyhemoglobin and 0% HbO2 for pure deoxy-Hb) for the given constituent versus its deconvoluted value. As an example of the calibrated deconvolution process, Figure 3 displays a representative spectrum measured from the model system is deconvoluted into weighted percentages of the reference spectra (HbO2, deoxy-Hb). For comparison, we include a superposition spectrum determined by the deconvoluted spectra. When averaged over the entire spectral range, the measured and calculated spectra deviate by 3.6%.

**RESULTS AND DISCUSSION**

**Instrument Performance.** Although the LCTF is continuously tunable from 500 to 750 nm, the limited throughput at both extremes restricts its useful range to between 525 and 725 nm. The basic spectral characteristics over this interval were examined to better understand the capabilities and potential of the imaging system. The LCTF, when presented with linearly polarized, collimated white light, produces transmissions from 2.7% (at 525 nm) to 24.6% (at 725 nm) with an average transmission of 10.0%.
across the useful spectral range. While the throughput of the LCTF is relatively low, it is sufficient to acquire the high-fidelity reflectance images required by clinical studies. The wavelength-dependent bandwidth of the device was measured as the full width at half-height (fwhh) and determined from the measured transmission spectra. The fwhh ranges from 0.24 (at 525 nm) to 0.57 nm (at 725 nm) with an average 0.38 nm across the useful range of the LCTF. This bandwidth is more than sufficient to spectroscopically resolve HbO₂ from deoxy-Hb and, in future studies, to discriminate between more subtle spectral differences, such as differentiating HbO₂ from nitrosyl-hemoglobin in which the spectroscopic signatures differ by only 1 nm.

The LCTF is expected, in general, to achieve an optimal spatial resolution when utilizing a collimated light path as, for example, from an infinity-corrected optical microscope. The LCTF in our macroscopic mode is positioned in front of the camera lens and is exposed to uncollimated radiation. In this optical configuration, it is anticipated that the spatial resolution will be only slightly degraded, but it is sufficient for the macroscopic studies and detector pixel binning parameters typically presented in clinical usage.

The spatial resolution of the hyperspectral imaging device was examined by calculating the percent contrast for the system, both with and without the LCTF, while employing two different CCD binning parameters. Many considerations contribute to the overall spatial resolution of the system, including focal distance, f/stop, depth of field, type of camera lens, detector pixel dimensions, and degree of pixel binning. Adjustments in any of these parameters can cause changes in the spatial resolution ranging from the subtle to the dramatic. For this study, a conventional 50-mm camera lens at f/stop 1.4 and a focal distance of ~5.5 ft was employed to image a standard resolution target. The conditions were chosen to be similar to those expected to be used while in a clinical setting.

A detector pixel binning of 3 x 3 over the full 768 x 512 CCD chip, for a total image size of 256 x 170 pixels, was employed to increase the sensitivity and speed of data collection. The 3 x 3 binning resulted in a square "super pixel" that images a 0.63 x 0.63 mm area of the sample. Percent contrast measurements with the LCTF tuned to 532 nm and with a 532-nm band-pass filter in the beam path before the camera lens are shown in Figure 4A, where we note that the results for both the LCTF and a spectral limiting band-pass filter are comparable. As the spatial frequency approaches 0.63 line pairs/mm (as defined by the USAF 1951 resolution target), a deviation in the two plots can be seen. At this point, the spatial resolution of 0.79 mm [(0.63 (line pairs/mm))^(-1) x \(\sqrt{2}\)] has begun to approach the size of a single "superpixel", and therefore, the resolution becomes detector limited and no further improvement is expected. When the full chip is employed with no binning, each pixel corresponds to a 0.22 x 0.22 mm sample area, thus removing the binning limitation. The percent contrast collected at 532 nm for both the band-pass filter and the LCTF, during the unbinned condition, is shown in Figure 4B. The expected degradation in the spatial resolution due to the LCTF is observed. By applying Rayleigh's criterion, the spatial resolution of the system employing the LCTF is of the order of 0.45 mm and demonstrates that the imaging system has a spatial resolution more than sufficient for most clinical investigations.

The effect of skin pigmentation on the determination of the percentage of HbO₂ was investigated by examining data from the palmar surface of the hand of several individuals covering a wide range of skin tones. The palm, instead of the forearm, leg, or posterior of the hand, was chosen for analysis because of its relatively low melanin content. This reduced amount of melanin, the pigment responsible for skin tone, minimizes the effect that skin pigmentation may have on the measured reflectance spectra. While it has been noted that, in general, skin pigmentation can affect the spectral baseline and, therefore, the observed absorbance value,28 our sampling area and processing method minimizes these effects. Since the percentage of HbO₂ determined for our pigmentation test group had a variance of ±1% skin pigmentation was not a major contributor to the uncertainty of the palmar measurements.

**Vascular Dysfunction Model.** Vascular dysfunction relates to the impairment or failure of a network of blood vessels, which...
results in a decrease in blood perfusing a tissue. These circumstances may be the direct or indirect result of a multiplicity of diseases, such as atherosclerosis, hypertension, diabetes, or sickle cell anemia. A form of vascular dysfunction is ischemia, which connotes a reduced blood supply due to a mechanical obstruction or a narrowing primarily of the arteries. To simulate an acute or temporary ischemia, a blood pressure cuff was placed around a subject’s left arm and inflated above the individual’s systolic blood pressure, the blood pressure measured while the heart contracts within the cardiac cycle. Subsequently, when the cuff pressure is released, one observes and measures reactive hyperemia, the increased amount of blood flowing to an organ during tissue reperfusion. The visible reflectance imaging system easily performs the spatial and temporal measurements required for assessing the changes in the percentages of HbO₂ across a skin area during the various phases of first ischemia and then the subsequent reactive hyperemia, which occurs during reperfusion.

For the clinical measurements, a baseline hyperspectral image cube for both the experimental and control hands was acquired prior to inflating the blood pressure cuff. The cuff remained inflated at a supersystolic pressure of 140–150 mmHg (20–30% above the subject’s resting systolic blood pressure of 115 mmHg) before data collection and for 2 min during data collection. Upon completion of the occlusion measurement, the blood pressure cuff was deflated. Once the occlusion was released, blood filled the dilated vasculature, thus increasing the regional blood flow into the formerly occluded arm and hand. On the basis of blood flow studies in sickle cell patients, we hypothesize that the percentages of HbO₂ will oscillate during our stipulated conditions of proximal ischemia, followed by release of the occlusion and the resulting reactive hyperemia during reperfusion.

This simulation of acute ischemia with a subsequent reactive hyperemia was imaged as a function of time to produce the time series of images seen in Figure 5. The images within the figure are arranged according to the predetermined sampling time points of the experimental timeline (Figure 2). Each image was sampled as indicated by the square boxes, which were labeled L1–L4 (left hand, areas 1–4) and R1–R4 (right hand, areas 1–4) as shown in Figure 5. Each sampling box includes 100 pixels producing an average spectrum and its related percentage of HbO₂. These images visualize the percentage of HbO₂ distributed throughout the hand.

Figure 5A presents an image of the palmar regions of the hands prior to the induced vascular dysfunction, the baseline measure; the percentages of HbO₂ in the measured areas of both hands appears to be relatively similar; the brighter intensity implies an increased percentage of HbO₂ in the hand. The occlusion image, Figure 5B, shows a significant difference in pixel intensity, between the two hands reflecting differences in the percentages of HbO₂ between the two extremities. The percentage of HbO₂ in the occluded left hand (darker areas) is significantly less than the unoccluded right hand, and both hands exhibit decreased percentages of HbO₂ compared to the baseline images (Figure 5A). The first reperfusion image, Figure 5C, shows the experimental left hand is brighter than either the unoccluded hand or the baseline hand, characteristic of reactive hyperemia. During

![Figure 5](image_url)
subsequent reperfusion, Figure 5D–F, the percentage of HbO₂ returns to near-baseline levels. Although the blood pressure cuff was placed on the left arm, the right hand exhibits counter lateral effects (Figure 5B), which may indicate vasomotor activity responding to the regional occlusion, as well as a complete systemic response.

The average percentages of HbO₂ are plotted as a function of time, Figure 6, for each of the numbered regions in Figure 5. The plots illustrate that within the two hands a small spatial variability in the percentage of HbO₂ occurs. This may indicate oxygen exchange from hemoglobin to the tissue as arterial blood in the capillaries becomes venous. Averaging the results of the sampled areas (L1–4, R1–4), one notes that the left hand (the hand undergoing a vascular occlusion) exhibits a 20.3% decrease in HbO₂ (from the baseline of 79.9 ± 2.4% HbO₂, Figure 5A, to the occluded value of 63.7 ± 3.2% HbO₂, Figure 5B) during the occlusion measure, that is, during acute ischemia. Release of the cuff pressure, which permits reactive hyperemia, is followed by an increase in the percentage of HbO₂ (84.5 ± 1.6% HbO₂, Figure 5C), which is 5.5% greater than the baseline measure. The right hand, even though unoccluded, showed a 7.7% drop in the percentage of HbO₂ from baseline values of 81.5 ± 2.2% (Figure 5A) to 75.2 ± 3.2% HbO₂ (Figure 5B). This effect may be interpreted as a counterlateral effect of vasomotor control responding to regional acute ischemia induced by the blood pressure cuff, which also affects the systemic vasculature. During reperfusion, the percentage of HbO₂ in the unoccluded hand from the imaging data was 80.9 ± 2.6% (Figure 5C), a value returning toward baseline values. This observation suggests that reactive hyperemia is a regional effect, unlike the ischemia, which has regional and systemic vascular consequences. The remaining reperfusion measurements (Figure 5D–F) vary within the statistical standard deviation, indicating that the system is returning to homeostasis. These time-dependent measures of (a) a decrease in percentage of HbO₂ from a baseline level during occlusion, (b) a subsequent increase to and above baseline during reactive hyperemia, and (c) a return to baseline values during reperfusion illustrate the sensitivity of the technique to HbO₂ saturation and may allow us, in the future, to study in detail and to diagnose various forms of vascular dysfunction.

CONCLUSIONS

The basic concept of hyperspectral imaging has been widely used in both military and civilian applications in identifying "targets" from airborne or satellite platforms. In contrast, for medical applications our in vivo, visible reflectance, hyperspectral approach rapidly generates reliable images which provide spatially resolved chemical information in either a static or time-resolved mode. We have described and characterized an instrument for performing noninvasive, in vivo, macroscopic (17 by 17 cm field of view) visible reflectance (525–725 nm) hyperspectral imaging in a clinical venue. The 768 by 512 multichannel array detector approach provides an imaging capability, while the use of an LCTF allows high-resolution spectra to be recorded, resulting in sufficient data for applying statistical analyses over spectroscopically distinct domains. An integration of these technologies enables the practitioner to image anatomical features with submillimeter spatial resolution while visualizing chemical changes at the molecular level. The characteristics of the system reflect an average spectral bandwidth (fwhh) of 0.38 nm with an average transmittance of 10.0% and a spatial resolution of 0.45 mm. Spectra associated with each detector pixel are deconvoluted using a multivariate least-squares analysis; the resulting images reflect the percentages of HbO₂ within the sampled areas.

The multichannel array detector approach described provides sufficient data for applying statistical analyses over spectroscopically distinct domains. As a physiologic example, we modeled vascular dysfunction by applying pressure to an arm first to produce an acute ischemia, and then, by releasing the pressure, to measure the reactive hyperemia occurring during tissue
reperfusion. These conditions lead to an oscillatory behavior in blood flow. Furthermore, we note that the induced local, acute ischemia involves an entire systemic effect, while local reactive hyperemia affects only a regional area.

With present day computational power, one approaches a seamless transition in interpreting the spatial and spectral domains of acquired images. Because of the high spatial and spectral resolution intrinsic to the hyperspectral imaging approach and the ability to perform time-resolved measurements, we expect this technique to become a useful diagnostic tool in a variety of disciplines in addition to its use as a noninvasive, in vivo clinical technique.

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