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Use of a Blood Substitute to Determine Instantaneous Murine Right Ventricular Thickening With Optical Coherence Tomography

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Background—A satisfactory imaging technique to determine regional wall thickening of the murine myocardium is not available. Although cardiovascular imaging with light offers a novel solution, application is problematic because scattering by erythrocytes causes significant optical attenuation.

Methods and Results—Optical coherence tomography (OCT) is a technique for detailed resolution imaging of highly scattering biological tissues. To reduce the high level of blood scattering, a method was devised whereby murine blood was replaced with a hemoglobin-based blood substitute. The scattering and absorption properties of in vitro preparations of whole blood and dilutions of blood with a blood substitute were determined with a spectrophotometer and an inverse-adding doubling algorithm. OCT imaging of the same dilutions demonstrated a significant reduction in scattering at a hematocrit <5%. A fiber-optic OCT imaging system was used to image the murine right midventricular free wall before and after isovolumic replacement with blood substitute. Strong light attenuation prevented full thickness imaging before replacement, whereas visualization of the full ventricular thickness was possible after replacement. Baseline and imaging hematocrits were 52.4±3.8% and 3.7±1.2%, respectively. End-systolic and end-diastolic thickness values were 0.458±0.051 mm and 0.352±0.047 mm. Percent thickening fraction was 30.8±7.5%.

Conclusion—Optical imaging of the intact beating murine right ventricle was substantially improved by isovolumic blood replacement with a hemoglobin-based blood substitute. Although the current study has been directed toward imaging the murine heart, a blood substitute may be applied to various optical diagnostic and therapeutic techniques under investigation in cardiovascular medicine. (***Circulation***. 2002;105:1843-1849.)

Key Words: blood substitute ■ imaging ■ lasers ■ myocardium ■ tomography

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Use of a genetically altered murine model for cardiovascular research has many advantages. First, murine heart function is similar to that in humans. Second, modest resource requirements make the model attractive for studies that require large sample sizes. Third, maturation is readily attained over a short period of time. Finally, because the murine genome is well characterized and can be easily modified, cardiovascular molecular mechanisms can be investigated through gene alterations.

A reliable imaging technique to determine regional wall thickening in intact beating mouse hearts has not been realized. Conventional methods, such as ultrasound and MRI, are either too slow for murine heart rates, too expensive for everyday laboratory use, or do not provide sufficient spatial resolution for the small dimensions of the murine heart. A promising new technique, optical coherence tomography (OCT), may be an appropriate imaging modality for murine cardiovascular studies. OCT is analogous to ultrasound, measuring the intensity of backscattered light rather than sound waves. Because light travels faster than sound and has a substantially shorter wavelength, the use of OCT provides micron scale resolution[1] and faster-than-video imaging speeds.[2] Moreover, the relative simplicity of the instrumentation allows for inexpensive construction.

Early biomedical applications of OCT focused on imaging stationary and transparent tissues, such as the eye, in which imaging depths can be deeper than 2 cm.[3,4] Further developments have focused on nontransparent, highly scattering tissues, such as skin, in which structures as deep as 1 to 2 mm have been visualized.[5] Dynamic, but nearly transparent, structures such as the developing in vivo tadpole heart have also been studied with OCT.[6] Although Doppler OCT has been used to assess in vivo blood flow, the problem of blood attenuation was noted.[7] In as much as the murine right ventricle (RV) is a thin anterior chamber, the purpose of the current study is to determine

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whether OCT can be applied to a dynamic, nontransparent biological structure.

Recently, there has been increasing interest in applying OCT to the cardiovascular system. Intravascular imaging has been achieved in vivo in the rabbit aorta.[8] In vitro studies with coronary arteries suggest a method of determining fibrous cap thickness of atherosclerotic plaques.[9,10] Despite these recent successes, cardiovascular imaging with OCT remains problematic primarily because of the strong attenuation of light by blood. Light attenuation in blood originates from two sources: absorption by hemoglobin and scattering by red blood cells.[11–13] Kramer et al[14] showed that light attenuation in whole blood is 7× to 20× stronger than that in a solution with the same concentration of hemoglobin. The implication is that a significant source of light attenuation in whole blood is due to scattering. If the scattering properties of blood can be diminished, visualization of blood-containing structures, such as coronary arteries and ventricular myocardium, with optical imaging techniques may be feasible.

One approach to reduce light attenuation because of scattering is to modify the plasma to match the index of refraction to that of red blood cells.[15] We propose and demonstrate an alternative approach: the use of a hemoglobin-based blood substitute to lower the hematocrit and minimize scattering. This novel use of a blood substitute benefits from the biochemical properties of artificial oxygen carriers and takes advantage of their optical transparency at near infrared wavelengths. By sufficiently reducing the hematocrit with a blood substitute, we can sustain the intact beating murine heart and quantify regional right ventricular function.

Methods

OCT Instrumentation

OCT is an interferometric technique based on a broadband light source and coherent cross-correlation detection of light. The principles of OCT have been previously described.[1] Our OCT system is based on a single-mode optical fiber Michelson interferometer and a semiconductor amplifier light source. The source has a center wavelength of 1310 nm and a spectral bandwidth of 60 nm. To visualize light incident on the murine myocardium with the unaided eye, a wavelength division multiplexor was used to combine 630-nm red light with a 1310-nm source. A rapid optical scan delay line[16] enabled us to acquire an image of a dynamic tissue such as a beating murine heart at video rate. The axial and lateral resolutions were 16 μm and 12 μm, respectively. One A-scan (a depth scan) corresponded to 2 mm in air and comprised 5000 two-byte data samples, which corresponded to a time of 2.5 ms at 2 megasamples/s sampling rate. Labview software (National Instruments) was used to process acquired signals. A band-pass filter removed unwanted low- and high-frequency noise, and an incoherent demodulation method was used to compensate for nonlinearities in the galvanometer scanning.

In Vitro Murine Blood Studies

In vitro measurements were performed to determine the scattering and absorption properties of murine blood, blood substitute (Oxyglobin), and dilutions of murine blood with Oxyglobin. Oxyglobin is isotonic to blood (prepared in a modified lactated Ringer's solution) and should not result in any hemolysis. An additional study was performed to specify the hematocrit reduction required for adequate visualization of the full RV thickness. For both studies, dilutions of fresh murine blood with Oxyglobin (Biopure Corporation) were prepared to a hematocrit of 40%, 30%, 20%, 10%, 8%, 5%, and 3%.

Pure blood (hematocrit >40%) and pure Oxyglobin (hematocrit=0%) samples were also prepared.

A Cary 5E UV-Vis-NIR spectrophotometer (Varian) with a single integrating sphere was used for scattering and absorption measurements. Cuvettes that held 0.4 mL with an average path length of 0.72 mm were constructed with 2 microscope slides and cover slips. Cuvettes that contained the dilutions were placed in the spectrophotometer.

Spectral dependent transmittance and reflectance values were obtained from the spectrophotometer. An inverse adding-doubling program[17] developed in our laboratory was used to determine absorption (μ_a) and reduced scattering (μ_s') coefficients. The total attenuation coefficient of blood can be represented as the following: $\mu_{total} = \mu_a + \mu_s$.

Although μ_s and μ_s differ by a directional factor,[18] μ_s' is equivalent to the reciprocal distance between successive scattering events. Scattering is due to the presence of higher refractive index red blood cells in plasma whereas absorption is due to the presence of the chromophore hemoglobin.[11,13] Blood from 1 or 2 (pooled) mice was used for each sample (n=4). Measurements were recorded at 20°C.

To determine the effect of OCT signal attenuation caused by blood in the murine RV, dilutions were placed between 2 glass slides separated by a 0.15-mm air space. The 0.15-mm distance was selected as a compromise between obtaining sufficient signal amplitude from the lower surface at high hematocrit (eg, 45%) and a measurable attenuation at low hematocrit (eg, 5%). OCT images through the sample dilutions were recorded. Images were recorded for sample dilutions prepared at the hematocrits listed above.

Animal Protocol

The protocol was approved by the Institutional Animal Care and Use Committee at the University of Texas at Austin and San Antonio and conformed with *Guidelines for the Care and Use of Laboratory Animals* (NIH publication No. 86-23, revised in 1985) and *Principles of Laboratory Animal Care* (published by the National Society for Medical Research). Female C57-B6 mice (n=6; Jackson Laboratories, Bar Harbor, Me) that weighed 19 to 22 g were anesthetized by administering urethane (1000 mg/kg IP) and etomidate (25 mg/kg IP). Respiration was controlled through a tracheotomy cannula, and the animals were mechanically ventilated with 100% O_2 at 95 breaths per minute with a rodent ventilator (Harvard Apparatus Model 683). Needle electrodes were applied subcutaneously and connected to a microelectrode AC amplifier (Model 1800, A-M Systems) for ECG recording. The chest was entered by an anterior thoracotomy. An apical stab was made in the left ventricle with a 27-G needle, through which Oxyglobin was introduced into the bloodstream. A right neck cutdown was performed, and the right jugular vein was exposed. The right jugular vein was nicked for blood removal or compressed for hemostasis. Separate sites to infuse Oxyglobin and bleed the mouse were chosen so the intravascular exchange could be performed simultaneously. In this fashion, hemodynamic instability was minimized.

The OCT light source was aimed at the right midventricular free wall with the red aiming beam. Baseline images were recorded at the native hematocrit. Hematocrit was determined by centrifugation followed by comparison to a calibration chart. Oxyglobin was used to replace whole blood in a 1:1 fashion, in amounts of ≈300 μL at a time. This process was continued until a hematocrit of <5% was obtained. A 5% hematocrit threshold was determined from the in vitro slide study (see Results) and was consistent with previously documented studies.[11–13] Images were recorded as described above and processed offline.

Image Processing

To determine the epicardial and endocardial boundaries, gray-scaled images were converted to binary images. Application of 2-dimensional median, closed and open filters, was necessary to avoid speckling effects present in the binary image. Subtracting depth positions of the 2 boundaries yielded an instantaneous thick-
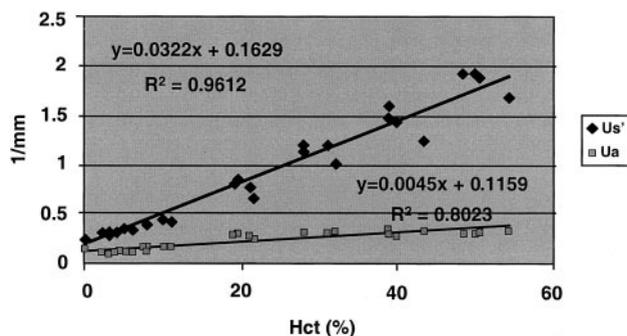
Figure 1. Optical properties of several dilutions of murine blood with Oxyglobin. The reduced scattering coefficient (μ_s') is dependent on the number of red blood cells per unit volume. The absorption coefficient (μ_a) depends on the concentration of hemoglobin. Hct indicates hematocrit.

ening fraction that was then low-pass filtered to derive a thickening versus time curve.

Because OCT measures optical path length, thickness was scaled according to a weighted average of the indices of refraction for myocardium and Oxyglobin. An average index of refraction of 1.343 for Oxyglobin was measured by comparing measured OCT thickness to the actual thickness.[19,20] A published refractive index value of human left ventricular myocardium (1.382 at 1300 nm) was used for murine myocardium.[19] End-diastolic thickness (EDT) was determined by noting the time of the R-wave peak on the surface ECG. End-systolic thickness (EST) was determined at the point of maximal thickness. Both EST and EDT values were averaged over 4 to 6 beats.

Statistics
A 2-tailed, Student's paired t test was used to compare the EDT and EST, baseline and Oxyglobin hematocrit, and baseline and Oxyglobin heart rates. Level of significance was taken as $P<0.05$. Data are presented as mean±SD.

Results
Figure 1 shows the results of scattering and absorption measurements using whole murine blood and dilutions with Oxyglobin. Optical properties were determined at the OCT source wavelength $\lambda = 1310$ nm. The scattering properties of whole murine blood decreased from 1.801±0.245 to 0.253±0.176 L/mm ($P<0.05$) when the hematocrit was reduced from physiological levels to <5%. In contrast, the absorption properties of whole murine blood were similar over the same range, decreasing from 0.344±0.127 to 0.129±0.118 ($P=$NS). The results imply that dilution of whole blood with Oxyglobin reduces primarily scattering of light at 1310 nm.

Figure 2 shows the results of in vitro determination of the effect of OCT signal attenuation caused by blood in the murine RV. A schematic of our experimental setup is shown in Figure 2A. Two microscope slides separated by a 150-μm air gap were assembled and contained various blood/Oxyglobin mixtures. The imaging beam was incident on the top microscope slide. Data are shown in Figure 2B. The slides appear further apart when light propagates through Oxyglobin compared with air. The greater apparent separation is due to the higher index of refraction of Oxyglobin, necessitating a longer time to traverse the same physical distance. The results in Figure 2B may be used to estimate the effect of

OCT signal attenuation caused by blood in the murine RV. Light attenuation in the intact murine RV with a fractional blood volume of 12.8%[21] and a hematocrit of 45% is nearly equivalent to that in the 5% image (ie, 0.12×0.45=5.4%) in Figure 2B. From the 5% image, we measured an attenuation of 0.214 (13.4 dB) over a 150-μm thickness. For a 450-μm thick RV murine myocardium,[22] expected attenuation by blood is 40.2 dB [(13.4 dB×450 μm)/150 μm]. The result indicates a substantial portion of the signal-to-noise ratio in the murine RV may be lost to scattering by blood. On the basis of this calculation and the results of pilot studies, in vivo imaging of the murine RV was performed at a hematocrit of <5%.

The Table shows thickness measurements from the OCT-imaged RVs after reduction of hematocrit to <5% in the intact beating murine heart. The heart rate in the presence of whole blood was 288±40 bpm at baseline and 312±42 bpm ($P=$NS) after Oxyglobin reduced the hematocrit to <5%. At the reduced hematocrit, the preparation was viable for several hours. The whole blood hematocrit was 52.4±3.8% at baseline and was reduced to 3.7±1.2% after replacement with Oxyglobin. The EDT of the RV free wall was 0.352±0.047 μm and the EST was 0.458±0.051 μm ($P<0.01$). These values were determined after reduction of the hematocrit because the RV endocardial boundary could not be identified in the presence of whole blood.

OCT images of the RV wall in Figure 3 are shown at baseline in the presence of whole blood and after reduction of the hematocrit with Oxyglobin. The epicardial boundary is easily delineated in both baseline and low hematocrit images. However, the endocardial boundary is only visible in the low-hematocrit images. Epicardial motion was observed to increase at low hematocrit in several of the images.

Figure 4 shows thickening versus time. Wall thickness measurements and surface ECGs from all 6 mice are shown. Thickness measurements were only available after hematocrit reduction with Oxyglobin. Each measurement was recorded over 1 second. The peak of the R-wave of the ECG occurs at the thinnest portion of the thickening curve in every image, as expected. The percent thickening for the group was 30.8±7.5% (Table 1).

Discussion
To the best of our knowledge, this study demonstrates for the first time that OCT can be used in conjunction with a blood substitute to measure instantaneous murine RV myocardial thickening. The problem of light scattering because of blood has not been overcome to date. Two approaches have been tested to reduce scattering by blood. A previous study increased the refractive index of plasma to approach that of red blood cells.[15] Another approach is to reduce attenuation due to scattering by lowering the hematocrit in the optical path. The latter approach has been used previously in the form of a saline bolus injection.[8] Because a saline bolus reduces oxygenation and only allows imaging for a short but variable time duration, a different approach is needed to prevent ischemia.

We have taken the approach of replacing red blood cells with a modified hemoglobin-based blood substitute, thereby removing the scattering objects and maintaining oxygenation.
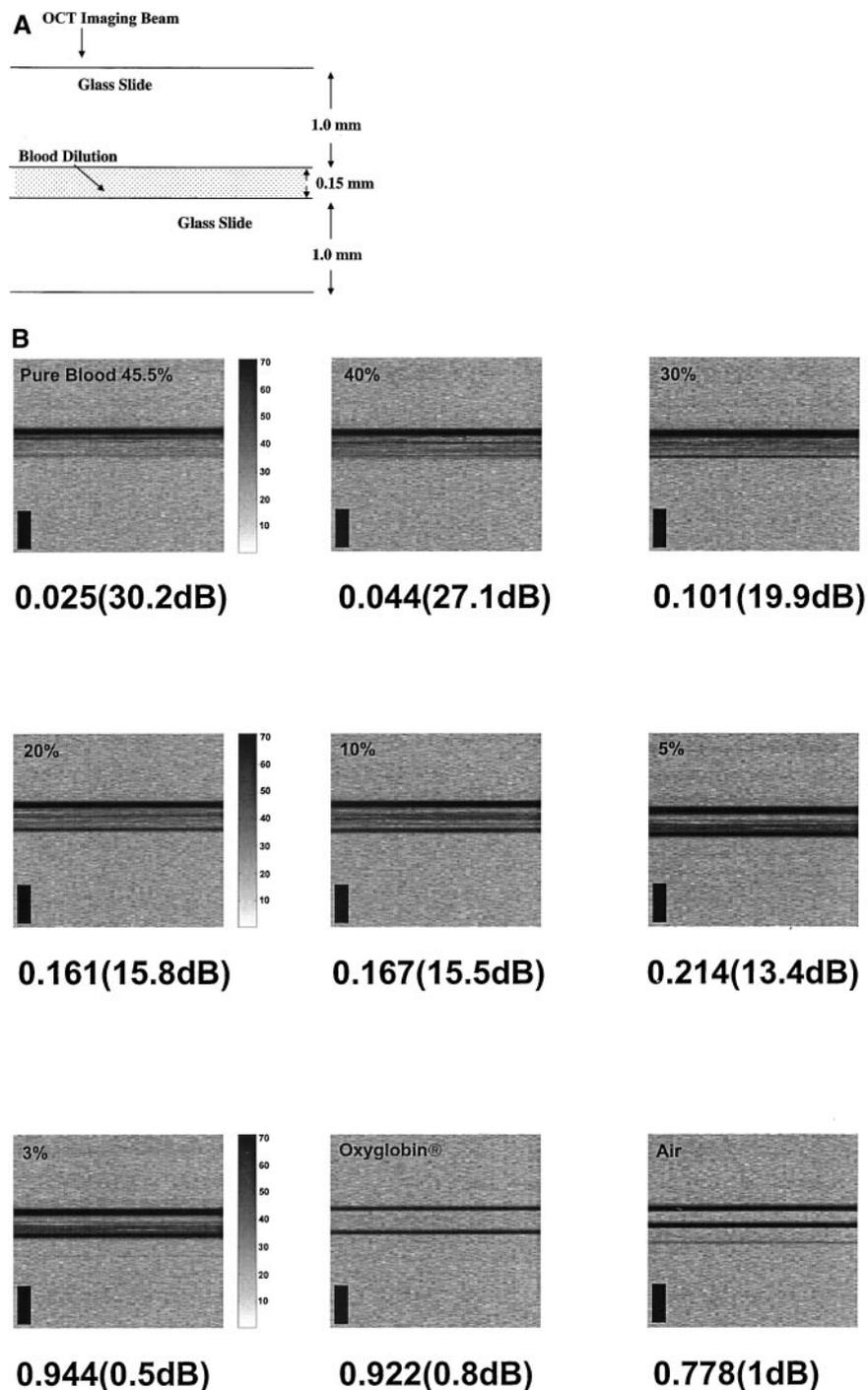
Figure 2. A, Diagram of slide assembly used to record OCT images of blood dilutions. B, OCT images of dilutions between slides. The hematocrit of each dilution is indicated in each figure. Signal attenuation at the lower interface (relative to the upper surface) is indicated below each figure as a ratio and in decibels. Scale bar is 0.30 mm.

Because modified hemoglobin blood substitutes are on the verge of gaining FDA approval,[23] regular use of such substances for many surgical and diagnostic procedures is becoming feasible. Though total volume replacement was not anticipated during the development of blood substitutes,[24] the physiological impact of such an exchange transfusion (down to ≈2% hematocrit) have been determined in sheep.[25] Postreplacement hemodynamics were stable, and animals survived with eventual regeneration of erythrocytes. Similarly, our murine creparation was viable for hours after a reduction of hematocrit to 3.7±1.2%.

For OCT imaging, light must penetrate the structure being studied. Light attenuation derives from both scattering and absorption. Results presented in Figure 1 indicate that red blood cells are a significant source of scattering, and a marked reduction in hematocrit is required (Figure 2B) for light to penetrate the distance needed to image the intact murine RV. These data confirm the prior work of Twersky,[11] Roggan et al,[12] and Steinke and Sheppard.[13] We found reduced light attenuation due to scattering could be accomplished through the use of Oxyglobin blood substitution. Future studies with artificial hemoglobins will allow the

RV End-Systolic and End-Diastolic Thicknesses and Thickening Fraction After Isovolumic Blood Replacement With Blood Substitute in Mice (n=6)

	Heart Rate, bpm	EDT, mm	EST, mm	%TF	Hematocrit
Baseline	288±40	NA	NA	NA	52.4±3.8%
After replacement	312±42	0.352±0.047*	0.458±0.051	30.8±7.5	3.7±1.2%

*$P<0.001$, EDT vs EST.
Measurements were not possible before replacement. Values are presented as mean±SD.
%TF indicates thickening fraction.

maintenance of physiological properties of the heart during OCT imaging.

The slight but nonsignificant decrease observed in absorption as a function of hematocrit requires further comment. Our working hypothesis is that the effect is due to a decrease in hemoglobin concentration. Typical physiological values for hemoglobin concentration are from 134 to 173 g/L.[12] According to manufacturer specifications, Oxyglobin has a hemoglobin concentration of 130 g/L, which indicates a concentration decrease in the replacement process. Clearly, however, decreased absorption was a much smaller factor in signal improvement than was scattering over the range of hematocrit examined.

Table 1 presents RV thickening measurements after the incorporation of the blood substitute. Previously published results[22] of mean RV thickness measured with transesopha-geal echocardiography and MRI (0.41±0.11 mm and 0.46±0.10 mm, respectively) agree closely with our values. Attempts to quantify RV thickening fraction (% thickening fraction) in other mammals yielded 25% in humans[26] and 14.1% in the canine RV.[27] These results are also similar to those found for the murine RV in the current study.

Possible sources of error in thickness measurements include an imprecise geometry between the beam and myocardium. During a heartbeat, some solid body rotation occurs so the instantaneous measured thickness will be different from the true thickness by the cosine of the angle between the normal of the heart surface and the beam (eg, 0.4% for a 5° deviation). Also, the thickness value depends on the average index of refraction used to convert measured optical path length to physical thickness. We used a weighted average of refractive index between myocardium and the blood substi-
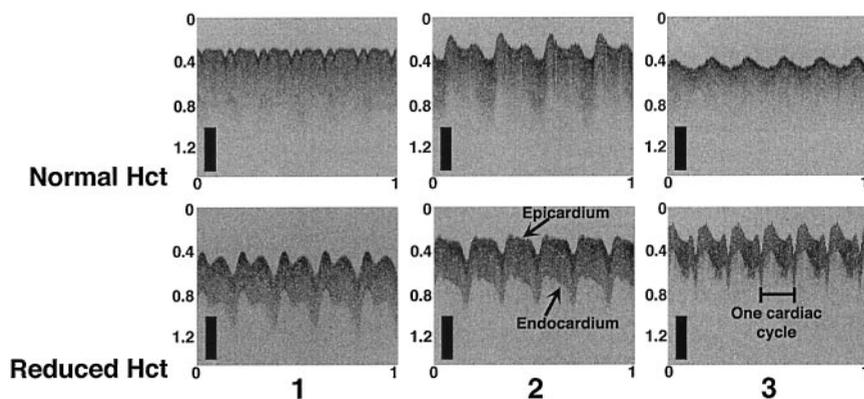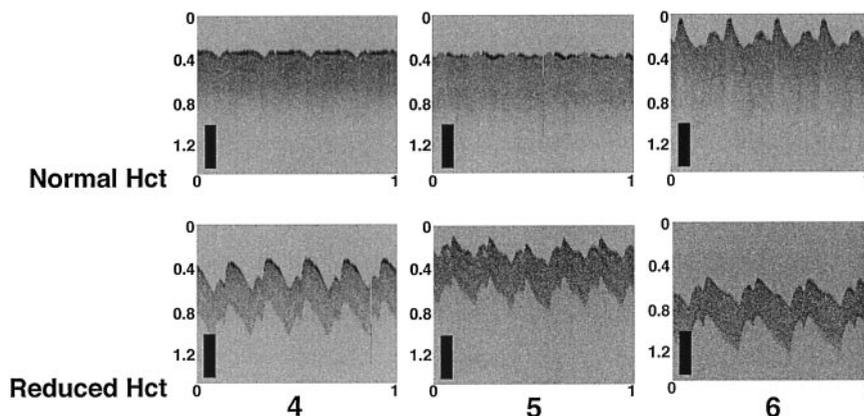

Figure 3. OCT images of the murine RV at baseline and after reduction to <5% hematocrit (Hct) with Oxyglobin. Scale bar is 0.40 mm. Horizontal axis is time in seconds.
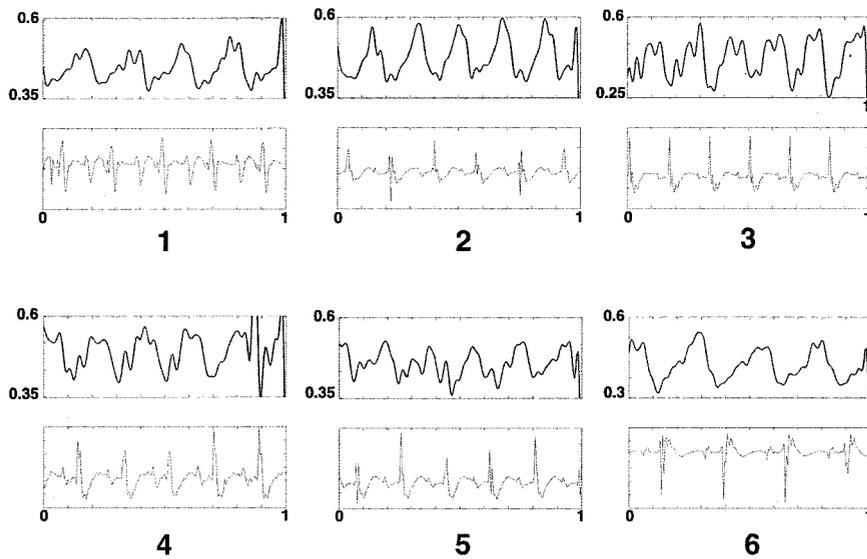
Figure 4. Instantaneous right ventricular wall thickening (top) and surface ECG (bottom) after reducing the hematocrit to <5% with Oxyglobin. Horizontal axis is time in seconds. Vertical axis is distance in mm.

tute.[28] In addition to the aforementioned errors, RV thickness varies along the axis of the chamber. Measurements made at the canine conus and apex differ significantly from those made at the midventricular free wall.[27] We attempted to visibly align the beam at the midventricular level, but the exact position may have deviated.

Imaging depths for OCT have been demonstrated at 1 to 2 mm in highly scattering tissues such as skin.[5] Consequently, one might expect a penetration depth greater than the thickness of the RV, which is near 0.45 mm.[22] Because the computed signal attenuation by blood in the murine RV is substantial (40.2 dB), expected signal attenuation (double pass) from the endocardium is severe and identification of the boundary is difficult. This was demonstrated experimentally. In Figure 3, a clear epicardial boundary is observed in whole blood images. Because the change in the refractive index of air ($n=1$) to myocardium ($n\approx1.38$) is large, we expected and observed a well-delineated epicardial boundary. OCT measures the intensity of back-scattered light that occurs at such a change in index of refraction. The contrast at the endocardial boundary, however, is substantially reduced. First, because our calculation indicates that severe light attenuation (40.2 dB) occurs with a double pass through the myocardium, the back-reflected signal amplitude is substantially reduced. Second, the index of refraction change at the endocardium/blood boundary is small compared with the air/epicardium boundary. The refractive index of myocardium is much closer to blood than air. Only after hemodilution with Oxyglobin to 5% hematocrit was light attenuation reduced sufficiently so that the full thickness of the RV could be imaged.

Because blood does not account for the majority of myocardial volume (12.8%, reference 21), one might be surprised that diminishing only the scattering coefficient of the blood would result in such a dramatic improvement in RV-imaging depth. The expected attenuation due to scattering of OCT light by blood can be estimated from the results presented in Figure 2B. As described in Results, the expected attenuation from blood alone in a 450-μm thick murine RV[22] is $\approx$100 times (40.2 dB). Because the reflection amplitude

from the inner myocardial wall of the RV will be less than that from the glass-liquid boundary in Figure 2B, we expect the signal amplitude (from the endocardium) will be reduced >100× relative to the air-myocardial interface. Taken together, these results indicate that substantial attenuation due to scattering by blood may be neutralized by reducing the hematocrit. With this technique, one can increase signal amplitude sufficiently to reveal the full thickness of the murine RV myocardium in OCT images.

Many investigators have recognized the potential advantages of applying optical techniques to diagnose and treat cardiovascular diseases. For example, optical techniques have been applied to assist in passing a guidewire through total coronary occlusions in interventional procedures.[29] Photodynamic therapy is being investigated as a potential approach to effect a cytotoxic response in atherosclerotic plaque.[30] Inasmuch as spectroscopic methods are capable of providing biochemical-specific information on tissue composition, reflectance and Raman techniques have been applied to cardiovascular diagnostics with various degrees of success.[31–33] More recently, OCT has been used to record depth-resolved images of fibrous lesions on the surface of coronary arteries.[9,10] A limitation of these optical techniques is the strong attenuation due to scattering by blood that is frequently positioned between the probe and targeted tissue structure. On the basis of the results of the present study, the application of these techniques may be better facilitated by the use of a blood substitute with a scattering attenuation coefficient less than that of blood. An alternative class of agents, perfluorocarbon emulsions, has been proposed as a blood substitute.[24] Because the emulsions contain particles, however, light attenuation due to Rayleigh scattering may reduce the back-reflected signal amplitude.

In conclusion, a hemoglobin-based blood substitute was used to substantially improve optical-imaging conditions in the blood-filled tissues of the murine RV. Imaging was possible because the light attenuation due to blood scattering was significantly diminished. The use of a hemoglobin-based blood substitute may offer a solution to light scattering due to

blood in applications of OCT to the in vivo cardiovascular system.

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