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Optical Doppler Tomography: Imaging *in vivo* Blood Flow Dynamics Following Pharmacological Intervention and Photodynamic Therapy

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ABSTRACT

A noninvasive optical technique has been developed for imaging *in vivo* blood flow dynamics and vessel structure with high spatial resolution. The technique is based on optical Doppler tomography, which combines Doppler velocimetry with optical coherence tomography to measure blood flow velocity at discrete spatial locations in turbid biological tissue. Applications of this technique for monitoring changes in blood flow dynamics and vessel structure following pharmacological intervention and photodynamic therapy are demonstrated.

INTRODUCTION

Noninvasive techniques for imaging *in vivo* blood flow are of great value for biomedical research and clinical diagnostics (1) where many diseases have a vascular etiology or involvement. In dermatology, for example, the superficial dermal plexus alone is particularly affected by the presence of disease (*e.g.* psoriasis, eczema, scleroderma), malformation (*e.g.* port-wine stain, hemangioma, telangiectasia) or trauma (*e.g.* irritation, wound, burn). In these situations, it would be most advantageous to the clinician if blood flow and structural features could be isolated and probed at user-specified discrete spatial locations in either the superficial or deep dermis. Localized blood flow monitoring is also critical for reconstructive procedures involving rotational or free flaps where vascular occlusion occurs in about 5–10% of cases (2). Early recognition of vascular compromise is essential for the salvage of failing flaps and replants because the effectiveness of intervention is inversely related to the time of ischemia (2). Numerous approaches for blood flow monitoring have been investigated including conventional and magnetic resonance angiography, laser Doppler flow-

metry (LDF)[†] (3), and Doppler ultrasound (4). However, a noninvasive technique for *in vivo* blood flow imaging with high spatial resolution is currently not available as a diagnostic tool in clinical medicine. Conventional LDF, for example, has been used to measure mean blood perfusion in the peripheral microcirculation. Strong optical scattering in biological tissue, however, limits spatially resolved flow measurements by LDF. Although Doppler ultrasound imaging provides a means to resolve flow velocities at different locations in a scattering medium, the relatively long acoustic wavelength required for deep tissue penetration limits spatial resolution to approximately 200 μm .

Localized flow velocity detection with high spatial resolution can be achieved by using coherence gating (5). Optical Doppler tomography (ODT) (6–9), a noninvasive optical technique, combines LDF with optical coherence tomography (10–12) to obtain high-resolution tomographic images of static and moving constituents in highly scattering biological tissues. Using a Michelson interferometer with a low coherence light source, ODT measures the amplitude and frequency of the interference fringe intensity generated between reference and target arms to form structural and velocity images. High spatial resolution is possible because light backscattered from the sample recombines with the reference beam and forms interference fringes only when the optical path length difference is within the coherence length of the source light. When light backscattered from a moving constituent interferes with the reference beam, a Doppler frequency shift occurs (Δf_D) in the interference fringe:

$$\Delta f_D = \frac{1}{2\pi}(\mathbf{k}_s - \mathbf{k}_i) \cdot \mathbf{v} \quad (1)$$

where $\mathbf{k}_i$ and $\mathbf{k}_s$ are wave vectors of incoming and scattered light respectively, and $\mathbf{v}$ is the velocity of the moving particle. With knowledge of the angle between $(\mathbf{k}_s - \mathbf{k}_i)$ and $\mathbf{v}$, measurement of the Doppler frequency shift (Δf_D) allows determination of particle velocity at discrete user-specified locations in a turbid sample.

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[†]Abbreviations: AID, analog to digital; BPD, benzoperphyrin derivative; CAM, chorioallantoic membrane; LDF, laser Doppler flowmetry; NO, nitric oxide; ODT, optical Doppler tomography; PDT, photodynamic therapy; SLD, superluminescent diode; STFFT, short-time fast Fourier transform.

Figure 1. Schematic of ODT instrumentation.

We describe in this paper the development of an ODT system for noninvasive *in vivo* imaging of blood flow dynamics and tissue structures with high spatial resolution in the chick chorioallantoic membrane (CAM) and rodent mesentery. Potential clinical applications of this technique to monitor changes in flow dynamics and vessel structure after application of a vasoactive drug and photodynamic therapy (PDT) are demonstrated.

MATERIALS AND METHODS

ODT instrumentation. ODT (Fig. 1) uses a fiber optic Michelson interferometer with a superluminescent diode (SLD) ($\lambda_0 = 850$ nm, $\Delta\lambda_{FWHM} = 25$ nm) as the light source. The reference mirror and sample constitute the two arms of the interferometer. Light from the SLD and an aiming beam (He-Ne laser, $\lambda = 633$ nm) is coupled into a fiber interferometer using a 2×1 coupler and then split equally into reference and target arms by a 2×2 fiber coupler. Piezoelectric cylinders are used to modulate the optical path lengths of light in the reference and target arms by stretching the fiber wrapped around cylinders. A ramp electrical wave (80 Hz) is used to drive the piezoelectric cylinders and generate optical phase modulation for the interference fringes ($f_0 = 1600$ Hz). Light in the sample path is focused onto the turbid sample by a gradient index lens (NA = 0.2) with the optical axis oriented at $15\text{--}20^\circ$ from the sample normal. The ODT velocity and structural images are obtained by sequential lateral (x direction in Fig. 1) scans of the sample probe at constant horizontal velocity (800 $\mu\text{m/s}$) followed by longitudinal (z direction in Fig. 1) incremental movement along the surface normal.

To maintain the coherence gate at the beam waist position in the turbid sample, a dynamic focus-tracking technique is used, where for each incremental movement (δ_1) of the sample probe along the surface normal, the reference mirror is translated (δ_2) to compensate for the new beam waist position in the turbid sample. By requiring the coherence gate to be at the position of the beam waist, a relationship between δ_1 and δ_2 is derived from geometrical optics (6),

$$\delta_2 = \delta_1(\bar{n}^2 - 1) \quad (2)$$

where $\bar{n}$ is mean refractive index of the turbid sample. Dynamic focus-tracking not only maintains lateral spatial resolution when probing deeper positions but also increases signal-to-noise ratio.

Light backscattered from the turbid sample is coupled back into the fiber and forms interference fringes at the photodetector. Temporal interference fringe intensity ($\Gamma_{ODT}[\tau]$) is measured by a single element silicon photovoltaic detector, where τ is the time delay between light from the reference and sample arms and is related to the optical path length difference (Δ) between the two by $\tau = \Delta/c$, where c is the speed of light. High axial spatial resolution is possible because interference is observed only when τ is within the source coherence time τ_c , or equivalently, when Δ is within the source coherence length ($L_c = \tau_c c$). The interference fringe signal is amplified, band passed, digitized (20 kHz) with a 16-bit analog-to-digital (A/D) converter and transferred to a computer workstation for data processing.

A spectrogram of the interference fringe intensity at time delay τ_i and frequency f_m is calculated using a short-time fast Fourier transform (STFFT):

$$\hat{\Gamma}_{ODT}(\tau_i, f_m) = |\text{STFFT}[f_m; \Gamma_{ODT}(\tau_i)]|^2 \quad (3)$$

A spectrogram is an estimate of the power spectrum of the temporal interference fringe intensity ($\Gamma_{ODT}[\tau_i, f_m]$) in the i th time window ($\tau_i, \tau_i + \Delta\tau_0$), where τ_i is the delay between light in sample and reference

arms, which determines the depth position (z_i) to be probed in the sample, and $\Delta\tau_0$ is the duration of the time window.

A tomographic structural image is obtained by calculating the backscattered light intensity, which is proportional to the value of the spectrogram at the phase modulation frequency (f_0). Because magnitude of the temporal interference fringe intensity decreases exponentially with increasing depth in the turbid sample, a logarithmic scale is used to display the ODT structural images:

$$S_{ODT}(i) = 10 \times \log[\hat{\Gamma}_{ODT}(\tau_i, f_0)] \quad (4)$$

Fluid flow velocity at each pixel is calculated by the Doppler frequency shift (Δf_D), which is determined by the difference between the carrier frequency established by the optical phase modulation (f_0) and the centroid (f_c) of the measured spectrogram at each pixel:

$$V_{ODT}(i) = \frac{\lambda_0 \Delta f_D}{2 \cos(\theta)} = \frac{\lambda_0 (f_c - f_0)}{2 \cos(\theta)} \quad (5)$$

where we have assumed, $\mathbf{k}_s = -\mathbf{k}_i$, θ is the angle between $\mathbf{k}_i$ and $\mathbf{v}$ in air and λ_0 is the vacuum center wavelength of the source. The centroid of the measured power spectrum at each pixel is given by:

$$f_c = \frac{\sum_m f_m \hat{\Gamma}_{ODT}(\tau_i, f_m)}{\sum_m \hat{\Gamma}_{ODT}(\tau_i, f_m)} \quad (6)$$

Lateral spatial resolution of our ODT instrument is limited by the numerical aperture of the target beam to 5 μm . Axial spatial resolution is limited by source coherence length (L_c) to 13 μm . We have imaged blood flow in an *in vivo* vessel with diameter as small as 25 μm in intact rodent skin (7). Higher axial resolution may be achieved by using a low coherence source with greater spectral bandwidth. Velocity resolution in our instrument (100 $\mu\text{m/s}$) is dependent on pixel acquisition time and the angle (θ) between flow velocity ($\mathbf{v}$) and the incoming light direction ($\mathbf{k}_i$). Velocity resolution may be improved with a smaller θ or longer pixel acquisition time. Using our instrument, the approximate time to record simultaneously ODT velocity and structural images is 3 min (e.g. $1 \times 1 \text{ mm}^2$, $5 \times 13 \mu\text{m}^2$ resolution).

In vivo Models. The CAM is a well-established model for studying the microvasculature and has been used extensively to investigate the effects of vasoactive drugs as well as optical and thermal processes in blood vessels (13). Fertilized chick eggs (Hyline W36 white leghorn) were washed with 70% alcohol and incubated at 37°C in 60% humidity. On days 3–4 of embryonic development, a hole was drilled through the shell apex and 2–3 mL albumin aspirated from the egg to create a false air sac. On the following day, a round window of 2 cm diameter was opened at the shell apex. The window was covered with a petri dish and the egg incubated for an additional 2 days. The egg was then removed from the incubator and put in a heat block filled with glass beads. The CAM vessels were located and imaged by ODT through the open window in the egg. The ODT images were recorded before and after topical application of a vasoactive drug, nitroglycerin.

The rodent mesentery is a useful model to demonstrate the potential applications of ODT for *in vivo* imaging of vascular blood flow in different organs. A rodent (*Rattus norvegicus*) was anesthetized with ketamine and xylazine. A 2 cm incision was made in the abdominal wall and a loop of small intestine exposed through the incision to allow access to the mesenteric vasculature. Isotonic saline was periodically applied to the exposed tissue to prevent desiccation. A PDT photosensitizer, benzoporphyrin derivative (BPD) solution, was injected (2 mg/kg) into the tail vein. Twenty minutes after photosensitizer injection, a semiconductor laser ($\lambda = 690$ nm, $D = 12 \text{ J/cm}^2$) was used to irradiate the mesentery for 120 s. Blood flow in the mesenteric artery was imaged before, 16 and 71 min after laser irradiation.

RESULTS AND DISCUSSION

ODT images of *in vivo* CAM blood flow

The ODT images of *in vivo* CAM blood flow are shown in Fig. 2. The vessel wall and membrane are evident in the structural image (Fig. 2A). Velocity images of blood flow moving in opposite directions, as determined by the sign of the Doppler shift (Δf_D), are shown in Fig. 2B and C, re-

Figure 2. The ODT images of *in vivo* blood flow in CAM. A, Structural image; B, velocity image of venous blood flow; C, velocity image of arterial blood flow; D, velocity profile along a horizontal cross section passing through the center of the vein, where the open circles are experimental data and the solid line is a parabolic fit.

spectively. Here static structures ($v = 0$) in the CAM appear dark, while blood flow in both the vein and artery appears as lighter shades. Magnitude of blood flow velocity at the center of the vein is maximal and decreases monotonically toward the peripheral wall. A horizontal cross section of the velocity image along the center of the vein is shown in Fig. 2D. The parabolic nature of the velocity profile indicates laminar flow in the CAM vein. In comparison to the vein, because of the pulsatile nature of arterial blood flow and lateral scan rate, velocity images of the artery appear discontinuous and show linear striated features (Fig. 2C). Diameters of the vein and artery are approximately 200 and 350 μm , respectively.

Effect of vasoactive drug on *in vivo* CAM blood flow monitored by ODT

To demonstrate the potential applications of ODT for *in vivo* blood flow monitoring after pharmacological intervention, the effect of a vasoactive drug on the CAM vasculature was studied.

Changes in arterial blood flow dynamics and vessel structure due to nitroglycerin are shown in Fig. 3, where Fig. 3A and B

Figure 3. Effect of vasodilating drug on blood flow in a CAM artery. The ODT structural and velocity images, respectively, before (A, B) and after (A', B') topical application of nitroglycerin.

are structural and velocity images, respectively, before, and Fig. 3A' and B' are corresponding images after topical application. The arterial wall can be clearly identified and dilation of the vessel after nitroglycerin application is observed in the structural images. Although velocity images appear discontinuous due to arterial pulsation (Fig. 3B and B'), enlargement of the cross sectional area of blood flow is evident. Peak blood flow velocity at the center of the vessel increased from 3000 to 4000 $\mu\text{m/s}$ after nitroglycerin application.

The effect of nitroglycerin on venous blood flow is shown in Fig. 4, where Fig. 4A and B are structural and velocity images, respectively, before, and Fig. 4A' and B' are corresponding images after topical application. Dilation of the vein due to nitroglycerin is observed in both structural and velocity images. In contrast to the artery, the peak velocity at the center of the vein decreased from 2000 to 1000 $\mu\text{m/s}$ after nitroglycerin application.

Nitroglycerin is a vasodilator used in the treatment of ischemic heart disease. The vasoactivity of nitroglycerin, as well as other nitrate compounds, arises from vascular metabolism to nitric oxide (NO) (14). Although the conversion of nitroglycerin to NO remains incompletely understood, a

Figure 4. Effect of vasodilating drug on blood flow in a CAM vein. The ODT structural and velocity images, respectively, before (A, B) and after (A', B') topical application of nitroglycerin.

Figure 5. The ODT images of *in vivo* blood flow in vasculature of rodent mesentery. A, Structural image; B, velocity image of venous flow; C, velocity image of arterial flow.

membrane-bound enzyme that involves sulfhydryl groups has been suggested (14). Once generated, NO activates soluble guanylate cyclase, which makes a second messenger cGMP within the vascular smooth muscle cell. This, in turn, suppresses intracellular calcium, which leads to vascular smooth muscle relaxation (15). It has also been observed that nitrovasodilators are venoselective irrespective of their mechanism of transformation, suggesting that NO itself may be venoselective *in vivo* (16).

Figures 3 and 4 indicate that the degree of CAM artery dilation is larger than the vein in response to nitroglycerin. This is probably due to the reversal of oxygenation in CAM vasculature where arteries and veins are oxygen poor and rich, respectively, because the embryo oxygenates itself from the surrounding air through the shell (13). In humans, *in vivo* vessels clearly show that NO is 10 times more potent on veins than arteries, whereas *in vitro* vessels show much less selectivity (16). The reason for this difference is unclear but might involve substances or cells in blood. The reversal of oxygenation could result in a reversal in selectivity, making nitroglycerin arterioselective in the CAM. However, determining the mechanism of vasoselectivity in CAM requires further investigations.

ODT images of *in vivo* rodent mesenteric blood flow

The ODT images of *in vivo* mesenteric blood flow are shown in Fig. 5. In the structural image (Fig. 5A), the attenuation of the backscattered light intensity is indicated by the decreased brightness of the image at deep positions. Two vessel-like structures, slightly flattened due to the effect of gravity, are evident. The region underneath the blood vessel appears dark in the structural image because of the strong at-

tenuation of light by red blood cells and Doppler shifting of the multiple scattered light out of the detection band established by the phase modulator. Velocity images of blood flow moving in opposite directions, as determined by the sign of the Doppler shift (Δf_D) are shown in Fig. 5B and C, respectively. Here static structures ($v = 0$) in the mesentery appear dark, while blood flow in both vein and artery appears as lighter shades. Blood flow in a large vein and a small artery with diameters of approximately 400 and 100 μm , respectively, is clearly identified.

The potential application of ODT for *in vivo* blood flow monitoring during PDT was investigated in rodent mesentery. Photodynamic therapy is a promising therapeutic modality for the treatment of malignant tumors (17) involving the use of a photosensitizing drug activated by light to cause selective tumor regression and necrosis. A number of possible tumor kill mechanisms have been suggested and investigated. Tumor cell killing by PDT is a complicated process and the mechanism remains incompletely understood (18,19). The most important cytotoxic agent is singlet oxygen produced by photoexcited sensitizers. Direct *in vitro* tumor cell kill *via* destruction of membranes and mitochondria by singlet oxygen was demonstrated (20). However, *in vivo* studies showed that PDT also affects the tumor microvasculature (18,21,22). It was suggested that oxygen-induced pathophysiological alterations in cell membranes, including the erythrocytes, led to vessel occlusion and hypoxia. Ultimately, vascular collapse and regional breakdown of O_2 and nutrient delivery caused tumor necrosis. The appearance of tumor ischemia and vasoconstriction, vessel blanching and stasis have been observed in PDT. These experiments used indirect methods (*e.g.* isotope-labeled microspheres) to measure blood flow and vessel diameter (23). The lack of a noninvasive method to characterize the blood flow and vessel structure simultaneously preclude a detailed investigation of blood flow dynamics during PDT. ODT offers a noninvasive method to provide not only velocity mapping of subsurface blood flow but also vessel structure changes following PDT.

To demonstrate the application of ODT for characterizing *in vivo* blood flow and vessel structure changes following PDT, mesenteric blood flow was studied after BPD injection and laser irradiation. ODT structural and velocity images were recorded before (Fig. 6A and A'), 16 (Fig. 6B and B') and 71 (Fig. 6C and C') min after laser irradiation. The diameter of the mesenteric artery was approximately 320 μm before laser irradiation. Sixteen minutes following laser irradiation, the diameter decreased from 320 μm to 60 μm ; 71 min after laser irradiation, the diameter increased to 385 μm . The results indicate that the artery goes into vasospasm after laser exposure and, subsequently, compensatory vasodilation occurs in response to PDT-induced tissue hypoxia. Monitoring the blood flow dynamics by ODT not only provides insight into understanding the mechanisms of PDT but also offers a potentially useful clinical tool to assess the effectiveness of treatment.

These results demonstrate that ODT offers a noninvasive method to image *in vivo* blood flow dynamics and surrounding tissue structure. The exceptionally high spatial resolution of ODT has broad implications for the clinical management of patients where microvascular blood flow monitoring is essential. Information provided by ODT can be used to determine tissue perfusion and viability before, during and af-

Figure 6. Blood flow dynamics and vessel structure change in rodent mesenteric artery after PDT. The ODT structural and velocity images, respectively, prior to laser irradiation (A, A'), 16 min after laser irradiation (B, B') and 71 min after laser irradiation (C, C').

ter surgical reconstructive procedures; assess the efficacy of pharmacological intervention for failing surgical flaps or replants; evaluate the skin microcirculation in a variety of lesions before, during and after treatment and investigate the mechanism of PDT for cancer treatment.

CONCLUSIONS

We have developed an ODT system for noninvasive imaging of *in vivo* blood flow. Tomographic velocity images of *in vivo* blood flow in the CAM and rodent mesentery were obtained using ODT. Applications of this technique to monitor *in vivo* blood flow dynamics and vessel structure changes in response to a vasoactive drug and PDT are demonstrated. ODT is noninvasive and noncontact, possesses exceptional spatial resolution and is a promising technique for both basic research and clinical medicine.

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