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Quantitative mapping of hemodynamics in the lung, brain, and dorsal window chamber-grown tumors using a novel, automated algorithm

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Abstract

Hemodynamic properties of vascular beds are of great interest in a variety of clinical and laboratory settings. However, there presently exists no automated, accurate, technically simple method for generating blood velocity maps of complex microvessel networks. Here we present a novel algorithm that addresses this problem by applying pixel-by-pixel cross-correlation to video data. Temporal signals at every spatial coordinate are compared with signals at neighboring points, generating a series of correlation maps from which speed and direction are calculated. User assisted definition of vessel geometries is not required, and sequential data are analyzed automatically, without user bias. Velocity measurements are validated against the dual-slit method and against capillary flow with known velocities. The algorithm is tested in three different biological models. Along with simultaneously acquired hemoglobin saturation and vascular geometry information, the hemodynamic maps presented here demonstrate an accurate, quantitative method of analyzing dynamic vascular systems.

INTRODUCTION

Vascular functionality is a key component in the study of many physiological systems wherein vascular flow characteristics are modulated by and/or indicative of oxygen availability, paracrine signaling, or metabolic demand. Investigation of such systems from a hemodynamic perspective provides insight into tissue function, as in studies of developmental vasculogenesis [1], cortical brain function [2], tumor hypoxia/angiogenesis dynamics [3], wound healing characteristics [4,5], etc. Blood flow analysis may also aid in the detection of a number of vascular pathologies wherein deficient oxygen/nutrient transport leads to tissue injury or accelerated malignant progression. For example, the eye particularly lends itself to such assessment due to the optical accessibility of its vasculature, and the incorporation of these data in clinical diagnoses is already commonplace [6]. In the early stages of disease, these pathological conditions may be most easily detected through

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the observation of aberrant hemodynamics. However, the ability to generate dynamic, highly-resolved blood velocity maps in a technically simple and automated manner has been an elusive goal, and the lack of such methods has restricted the widespread analysis of flow data in both laboratory and clinical applications.

Optical methods of measuring microvascular blood velocity have a long history due to their ability to non-invasively resolve hemodynamic properties at the level of single microvessels [7,8]. For decades, the dual-slit technique was the standard method for measuring blood velocity, and its application continues today due to its versatility, simplicity, and inexpensiveness [9]. This method uses dual acquisition windows to capture fluctuating optical signals at two points along a vessel. The peak of the cross-correlation between these two signals identifies the temporal delay between them from which velocity may be calculated. A magnified, video-rate recording of blood motion within the sample is all that is required in the data-collection phase. However, a drawback of this approach is the need for user-guided placement of signal acquisition windows for every vessel segment of interest in the data-processing phase. Thus, a complete observation of velocity characteristics within the observable vascular network would require the manual placement of numerous window pairs on vessels within the region of interest—a laborious process that is prone to selection bias.

These drawbacks have prompted the development of a number of other approaches to the analysis of video-based data, including a number of methods for high-resolution particle tracking [10–12]. Techniques that implement continuous velocity measurements along vessel centerlines can capture subtle variations in velocity patterns, at the cost of time-consuming manual vessel traces [13,14]. These techniques are insensitive to variations in speed across vessel profiles. The development of a semi-automated method, that tracks individual red blood cells through capillaries while simultaneously measuring the oxygen saturation of these cells, represented a major advance in the observation of microvascular oxygen transport [15]. This method requires user guidance, though, and may be limited to the analysis of capillary flow, where single cells can be tracked from frame to frame. Recently, an algorithm based on a cross-correlation analysis was demonstrated in a glioma model, providing impressive blood velocity maps [16]. However, this method processes temporal delays equivalent to only a single frame offset.

Thus it seems that video-based techniques, although technically straightforward, have inherent limitations, and are not ideal for the study of complex networks with large numbers of vessel segments. When investigating time-dependent changes in blood velocity patterns, these limitations restrict the ability to obtain and analyze spatial and temporal information on flow in vascular networks due to the sheer quantity of manual interactions required.

As an alternative to video-based methods, a number of spectroscopic techniques have recently emerged as a result of improvements in optical technology, computational efficiency, and digital signal acquisition/processing. Laser Doppler velocimetry is commonly used to extract volumetric blood velocity data in optical settings where individual vessels cannot be resolved [17]. Within optically accessible tissues, optical coherence tomography (OCT) is perhaps the most widely used spectroscopic technology, and its incorporation in the clinic as an ocular imaging technique has proven a valuable diagnostic resource. Even with the incorporation of Doppler-based calculations of velocity, however, long acquisition times and intensive computational analysis limit its ability to easily detect and quantify rapidly changing or unstable velocity dynamics. Due to insensitivity to movement perpendicular to the beam direction, Doppler calculations may be missing or inaccurate for a number of vessels, especially in samples where the vascular network is largely planar [18]. Laser speckle imaging may partially alleviate these drawbacks with

faster acquisition times and less directional sensitivity, but like OCT, this method cannot easily extract complete direction information [19]. Furthermore, these methods require expensive and technically challenging optical setups.

To address these challenges, we set out to create an inexpensive, easily implementable and fully automated method for analyzing functional dynamics of vascular networks. Here we present an algorithm for the extraction of hemodynamic properties from video images that does not require complex optical technology, generates easily interpretable and accurate speed and direction maps, and eliminates the need for human guidance, thus avoiding user bias in data analysis. Furthermore, in many optical settings it is capable of extracting velocity data from the movement of red blood cells alone, allowing for optical detection using either endogenous or artificial tracers, and ensuring consistency between experiments [20]. This efficient method of processing video sequences facilitates the investigation of large (both spatially and temporally) data sets, as demonstrated here through the generation of dynamic blood velocity movies.

The algorithm was applied to three model systems. The tumor-bearing murine dorsal window chamber was used to validate this technique and establish comprehensive models of angio-adaptation in the tumor microenvironment. A lung optical window was used to investigate the functional and structural characteristics of the pulmonary vascular network. Finally, datasets were acquired from the non-human primate brain during the course of electro-stimulation, producing dynamic sequences of hemodynamic responses which show the relationship between pial blood flow and neural activity.

METHODS

Dorsal window chamber surgery and tumor inoculation

A thorough description of the dorsal window chamber surgical procedure is provided in a prior publication. Briefly, nude mice were anesthetized using a ketamine/xylazine preparation and the surgical area was disinfected using exidine and ethanol. In each mouse, a circular incision 12mm in diameter was made in the loose dorsal skin, and the skin and underlying connective tissue were removed completely. This provided access to the dermal tissue on the opposite side. The incision was aligned with a hole cut into a specially constructed titanium frame, which was then sutured onto the animal to form a stable window into the underlying tissue.

Approximately 2×10^4 murine mammary carcinoma cells suspended in 20 μ L of serum-free media were injected beneath the exposed fascial plane. A sterile glass coverslip was then inserted into the titanium frame to form an air-tight, optically-accessible window into the inoculated tumor and surrounding tissue.

After the completion of the surgical procedure, antibiotic ointment was applied to any exposed wounds, and the animal was allowed to recover on an isothermal water circulation pad before returning it to its cage. After all surgeries were completed, the animals were returned to their housing facility and monitored daily for signs of surgical complication. After one week, most implanted cells grew to form viable tumors 1–2mm in diameter.

Video capture and registration

Videos of blood flow were collected in the dorsal window chamber using a standard scientific microscope (Zeiss Axio Observer.D1). The window was trans-illuminated with the broad spectrum halogen source. To enhance hemoglobin contrast, a liquid crystal tunable filter was set to 520 nm, a wavelength at which both oxy- and deoxy-hemoglobin display a high degree of optical absorption. This excluded light outside of the 515–525nm range. A 5x

objective (Zeiss FLUAR; 0.25 NA) was used to resolve discrete red blood cells as they moved through the vasculature. In its video capture mode, the CCD camera recorded 128 frames at 24 frames per second. Each frame was 1392×1040 pixels. The resultant video was saved as a multipage image file.

The video data were loaded into MATLAB as a three-dimensional matrix. The first two indices of the matrix represented the spatial coordinate of each pixel in a given frame, and the third, temporal index represented the frame number in the video sequence. At any particular spatial location, the temporal signal at that coordinate could be extracted by isolating the pixel grayscale value in each frame. For the spatial coordinate (i_0, j_0) , for example, the temporal signal is represented as a one-dimensional vector S_{i_0, j_0} . If the spatial coordinate (i_0, j_0) lies within a vessel, the temporal signal S_{i_0, j_0} fluctuates due to the movement of optically-absorbing red blood cells through that location.

Prior to velocity analysis, the video sequence often required frame registration. Small linear shifts between consecutive frames were often a result of the animal's respiratory and cardiac movements. In order to negate these effects, the video was processed frame-by-frame to find the relative horizontal and vertical pixel shifts that produced the smallest composite intensity differences between frames, $\min_{(m,n)}(\mathbf{R})$, where

$$R_{m,n} = \sum_{i,j \in Q} \left(F_{1(i,j)} - F_{2(i+m,j+n)} \right)^2$$

and

$$Q \equiv \mathbf{F}_1 \cap \mathbf{F}_{2(+m,+n)}$$

(where $\mathbf{F}_{2(+m,+n)}$ denotes indices in $\mathbf{F}_2$ offset by m rows and n columns relative to $\mathbf{F}_1$). For consecutive frames $\mathbf{F}_1$ and $\mathbf{F}_2$, $\mathbf{F}_2$ was shifted by $-m_0$ and $-n_0$, where m_0 and n_0 are the indices where $\mathbf{R}$ has its minimum value. Continuing this process throughout generated a registered image sequence. This sequence was represented as a three-dimensional matrix, $\mathbf{A}$, of size $M \times N \times P$. Where M and N are the number of rows and columns of pixels in each frame, and P is the total number of captured frames. In order to simplify future calculations, $\mathbf{A}$ was transformed into $\mathbf{A}'$, where the DC component along the temporal dimension was removed:

$$A'_{i,j,k} = A_{i,j,k} - \frac{1}{P} \sum_{k'=1}^P A_{i,j,k'}$$

Preliminary data masking

In the initial stages of analysis, a map was generated which indicated the degree to which pixels fluctuated coherently with their surrounding pixels. For a given spatial coordinate, (i_0, j_0) in $\mathbf{A}'$, the DC-subtracted temporal signal at that coordinate (S_0) was compared to that of each of the eight surrounding coordinates ($S_1 \dots S_8$). Element-wise products were calculated for corresponding elements pairs along the length of the two signals, and the total number of positive elements (representing signals increasing or decreasing together at a particular time) was assigned as a value for that particular coordinate:

$$R_{i_0,j_0} = \sum_{n=1}^8 \sum_{k=1}^P \{ \vec{S}_{0(k)} \cdot \vec{S}_{n(k)} > 0 \}$$

The derived coherence map, $\mathbf{R}$, was used to enhance efficiency by excluding the processing of pixels that did not fluctuate in synch with surrounding pixels (indicating they did not constitute an area of blood flow), based off of some threshold value for R_{i_0,j_0} . This map was also used as a color-intensity weighting factor for various visualization schemes.

Calculations of blood speed and direction

At any particular location (i_0, j_0) in $\mathbf{A}'$, blood speed and direction at that coordinate can be calculated (Figure 1). The coordinate's temporal signal, $\vec{S}_{i_0,j_0}$, is compared to the temporal signals in the surrounding area using a cross-correlation approach. Maximum values of the cross-correlation vector represent the temporal offset between the two signals which produces the best alignment. Thus, for the given coordinate, the newly generated, two-dimensional matrix $\mathbf{C}$ represents a local map of temporal offsets relative to the signal at (i_0, j_0) :

$$C_{i',j'} = \max_{(\tau)} (\vec{S}_{i_0,j_0} \star \vec{S}_{i',j'})$$

where $C_{i',j'}$ takes on the value of the frame shift (τ) that maximizes the cross-correlation term. (In order to limit the number of calculations, the ranges of i' and j' are typically limited to a few hundred pixels centered upon (i_0, j_0)). Figure 2 shows an example image of $\mathbf{C}$ taken from real data.

For computational efficiency, the cross-correlation operations were performed in the Fourier domain by employing the convolution theorem, which states that the Fourier transform of two convolved signals is equivalent to the product of their individual transforms. Therefore,

$$\vec{S}_{i_0,j_0} \star \vec{S}_{i',j'} = \mathcal{F}^{-1} \left[\mathcal{F}(\vec{S}_{i_0,j_0}) \cdot \mathcal{F}(\vec{S}_{i',j'})^* \right]$$

(Since we wished to calculate the cross-correlation of the signals rather than their convolution, one signal was reversed in time by taking the complex conjugate of its Fourier transform.)

After $\mathbf{C}$ was calculated, the algorithm identified large, spatially contiguous regions of common offset values within the matrix. Isolated values, or contiguous regions that contained less than the specified minimum number of elements, were ignored. For each offset value, the largest retained region was used for calculations of velocity. The Euclidian distance from centroid to centroid of incrementing regions represented the distance a given cluster of blood cells traveled from frame to frame. By measuring the direction and distance between these centroids, direction and speed can be calculated for the coordinate of interest. Pixels that failed to produce a given number of incremental offset regions were identified as non-flowing. This process was repeated for every pixel, generating complete speed and direction maps.

A slight Gaussian blur was applied to some displayed images to minimize visual noise.

Comparison of the mapping algorithm to the dual-slit technique

For validation, the algorithm was compared to the dual-slit technique. 2×2 binning was used to generate 44 Hz, 64-frame video sequences (each frame was 696×520 pixels). A total of 37 vessel segments were analyzed between two animals.

The dual-slit technique involves the placement of pairs of windows at upstream and downstream locations on a vessel segment. The intensity values of the pixels within the window were averaged at each time point to obtain a pair of temporal signals. The entire signal was then smoothed using a five-point sliding average operation. These signals were cross-correlated to find the temporal offset at which the two signals were best correlated. Dual-slit results were constrained to take on frame offsets representing realistic velocity values by limiting the range of temporal offsets to 1–18. By dividing the spatial distance between the two slits by this time delay, velocity values were obtained. The final velocity value for each segment represented the average velocity obtained from twelve independently placed window-pairs. This velocity was compared to twelve algorithm-derived velocity values calculated by averaging the individual values within the region spanning the area between the slit pairs.

The line representing the relationship between mapping algorithm and dual-slit values within common vessel segments was calculated using linear regression analysis. The mean and standard deviation of the twelve repeated measurements for each method were calculated, and from these values, the coefficient of variation was derived. The coefficient of variation over the 37 analyzed vessel segments were compared. A statistically significant difference in values between the two methods was demonstrated using the Wilcoxon rank-sum test.

Velocity validation using a vessel simulation

As a further validation of the mapping algorithm's accuracy, we calculated particle speed in a simulated vessel in which flow characteristics could be controlled (Supporting Video 2). These test data were generated by allowing randomly distributed, simulated particles to move down a column with a defined mean velocity, plus or minus a random lateral and longitudinal component at each step. Vessel width and particle density could be varied. Particle blur and optical noise were added. Various frame rates were simulated by taking the mean image over a sequence of frames to produce a single frame with the appropriate integration time.

Velocity validation using capillary tube measurements

In order to validate the accuracy of velocity measurements in a well-controlled physical system, videos of blood movement through a 500µm (inner diameter) heparinized capillary tube were analyzed. Whole blood was drawn from an anesthetized mouse and diluted 10:1 in heparinized saline. The dilution of the blood was necessary in order to overcome the optical attenuation through the capillary tube. The diluted blood was then injected through the capillary tube at a controlled rate using a syringe infusion pump.

For a given flow rate, the mean velocity through the capillary tube was calculated as

$$\bar{v}_1 = \frac{Q}{\pi \cdot r^2}$$

Here v_1 is in mm/s, the flow rate, Q , is in $\mu\text{L/s}$, and the tube radius, r , is in mm. A corresponding algorithm-derived mean velocity, v_2 , was determined from videos by averaging the calculated velocities at all pixels lying within the inner diameter of the tube.

v_1 represents the mean velocity over a paraboloid-shaped velocity cross-section, which describes flow through a three-dimensional cylinder. However, v_2 is the mean velocity over an imaging plane that bisects the vessel along its central axis. For an ideal paraboloid, such a section will have a mean value $4/3$ times greater than the mean value of the paraboloid over the area of the cylinder. Therefore, a scaling factor of $4/3$ must be applied to v_1 in order to directly compare it to v_2 .

Data acquisition in the pulmonary window

The pulmonary window was installed using methods described in detail elsewhere [21]. Since the breathing of the animal was controlled through artificial ventilation, respiration could be momentarily suspended for the few seconds required for video acquisition. Although this eliminated respiratory movement, cardiac movement was still a problem. This was corrected post acquisition using a registration algorithm that employed linear shifts to minimize the aggregate pixel-wise differences in intensity between consecutive frames. Pulmonary video sequences consisted of 64 frames collected at 44 Hz. Each frame was 250×250 pixels.

Data acquisition and statistical analysis of blood velocity in the primate brain

The surgical procedure for the exposure of the pial tissue for optical investigation is described in a prior publication [22]. Video sequences were acquired using a high-frame rate camera. The processing of these video data was complicated by the presence of significant cardiac and respiratory-associated movement in the video images. Relatively small frame sizes meant a registration algorithm that employed sub-pixel shifts was needed for accurate alignment across frames. This was achieved through the interpolation-based up-sampling of images. Frame registration was performed using the linear-shifting method mentioned above. The image sequence was then down-sampled back to the original resolution. This registration method provided superior image registration at the expense of a slight decrease in feature resolution due to the upsampling/downsampling process.

The full sequence of images spanned 8600 frames over 56 seconds, for a frame rate of 154 Hz. Each frame was 204×49 pixels. The mapping algorithm processed these in 64 frame clusters, each cluster separated by 4 frames. Mean velocity values were calculated over constant regions of interest. The dual-slit method (as described above) used 64 frame clusters, each cluster separated by 100 frames. While the mapping algorithm processed raw data, the dual-slit method operated on a smoothed signal (five-point sliding average) to improve the quality of calculations. The velocity values represent the average of 10 calculations using slit pairs which remained constant throughout the time course. Results from these two methods were each interpolated to produce 8600 velocity values. Comparisons between them are represented as correlations coefficients and p-values calculated using the Pearson product-moment method for linear relationships. Temporal correlations between the individual methods and arteriole diameter values were calculated using Spearman's method for non-linear relationships. Mean velocity values calculated during the baseline and recovery periods were statistically compared using a two-sample t-test.

Hemoglobin saturation and total hemoglobin calculations

The method of deriving hemoglobin saturation and total hemoglobin content is described in a prior publication, along with validation of the technique [23]. Briefly, in order to calculate

these parameters in window chamber models, a white light source was used to trans-illuminate the window chamber. A liquid crystal tunable filter was installed upstream of the camera to control the wavelength of transmitted signal incident upon the detector. After collecting a series of images at wavelengths between 500–600 nm in 10 nm increments, linear regression was used in the post-processing phase to extract hemoglobin parameters from the hyperspectral dataset (the optical absorption spectra of oxygenated and deoxygenated hemoglobin, along with a tissue scattering term, were provided as inputs).

Live Animal Use

All procedures involving live animals were performed in accordance with protocols approved by the Duke Institutional Animal Care and Use Committee. Every effort was made to avoid animal suffering, and appropriate anesthetics were used during all surgical procedures.

RESULTS

Calculations of hemodynamic parameters

The dorsal skin-fold window chamber was the main preparation used in developing this method [26]. This model provides a tissue plane that is easily accessible for high-resolution intravital imaging. Using a video-rate monochromatic camera attached to a conventional scientific microscope, the movement of blood through the sub-dermal vasculature was recorded. Visual analysis of these videos showed that intensity fluctuations at defined locations arose due to the heterogeneous clustering of red blood cells as they passed through vessels, and that these temporal signals showed a high degree of correlation along directions of flow. As these cells moved from one location to another, plots of temporal intensity fluctuations at the two positions maintained generally the same profile, but with a time-lag that depended on the velocity and the spatial distance between the two points.

These spatial-temporal correlations provided a means by which hemodynamic parameters could be estimated (Figure 1). The entire video sequence was first converted to a three-dimensional data structure in which temporal fluctuations at any given spatial coordinate were represented as a one-dimensional signal vector extracted from the matrix. For every spatial coordinate, the mapping algorithm implemented a series of pairwise comparisons between the signal at that coordinate to those in the local neighborhood. The compared signals were cross-correlated, and the frame-offset value that produced the best-aligned profiles was identified. For each coordinate of interest, image representations of these offset values showed clusters of incrementally increasing regions at locations lying along flow pathways (Figure 2). Speed and direction were implicitly represented within these data. By measuring the Euclidian distance from centroids of incrementally increasing clusters, the distance that cells traveled from frame to frame was calculated. This process was repeated for every spatial coordinate within the image sequence.

When applied to tumor-bearing dorsal window chamber videos (Supporting Video 2), this method generated high-resolution maps of velocity magnitude and direction (Figure 3a & 3b; Supporting Video 3 & 4). At 1392×1040 frame resolution over 128 frames, these maps required approximately 90 minutes to calculate in post-processing on a dedicated image processing computer (8-core, 3.40GHz CPU/8GB memory; 800MHz GPU/1GB memory), although this time varied according to vessel density. Other major parameters associated with micro-regional hemodynamics were simultaneously mapped; since changes in blood flow strongly influence oxygen delivery, hemoglobin saturation values within the same field of view were measured using methods previously described (Figure 3c) [23,26]. This

process was repeated over three days in order to track the evolution of these parameters over time (Supporting Figure 1–3).

Validation of hemodynamic maps

In order to test the accuracy of our velocity maps, independent measurements were required. We therefore employed the proven dual-slit technique to record velocity values from individual vessel segments and compared these results to those of the mapping algorithm. This analysis showed close agreement between the two methods for speeds up to 2 mm/s (Figure 4). The linear model for these data was $y = 1.0x - 0.015$, with $R^2 = 0.96$. The Bland-Altman plot of these comparisons showed a mean difference close to zero and 95% confidence intervals at approximately ± 0.2 mm/s (Supporting Figure 4). As velocities increased, measurements began to diverge due to increasing imprecision of the dual-slit method (Supporting Figure 5). To demonstrate this, we measured the coefficient of variation of repeated measurements for both techniques. This had mean values of 0.025 and 0.10 mm/s for the mapping algorithm and the dual-slit method, respectively, and standard errors were 0.0040 and 0.0097 mm/s. The variability among repeated measurements was significantly smaller for the mapping algorithm compared to the dual-slit method, with $p < 10^{-10}$.

In order to demonstrate the accuracy of the mapping algorithm against known velocities, we created a vessel simulation in which flow characteristics could be directly controlled. Algorithm derived velocities were plotted as a function of frame rate (Supporting Figure 6). We calculated the frame rate at which the algorithm-derived speed varied from the prescribed speed by more than 5%, and generated a linear fit from these data. These results showed 3.25 mm/s was the maximum accurate speed that could be calculated at 20 Hz, and for every 0.163 mm/s increase in velocity, one additional frame per second was needed to accommodate. Accuracy also decreased at a faster rate with particle density, although it was insensitive to variations in vessel diameter. By modifying particle blur to simulate deviations from the imaging plane, we observed that the algorithm is insensitive to variations in vessel depth, so long as the blurring of features does not prevent temporal fluctuations from being resolved. Although these data were derived from an idealized vessel and required frame rates for a given maximum velocity are likely larger for real data, these results provide a rough estimate of the operational range of a given system.

Data from capillary tube measurements provided a means of *in vitro* validation against known flow rates. Mean velocities for a given flow rate were compared to algorithm-derived data, and these showed a strong correlation (Supporting Figure 7). When plotted against one another, the linear fit for these data had $R^2 = 0.997$. The slope of this linear model was 1.32. After applying the 4/3 scaling factor (see methods), the Bland-Altman plot of these comparisons showed a mean difference near zero and 95% confidence intervals at approximately ± 0.2 mm/s (Supporting Figure 8).

Pulmonary window model

To test the approach under different conditions, we mapped blood velocity magnitude and direction in a pulmonary window chamber model. The procedure involves the surgical exposure of the rat lung for optical observation [21]. Using fluorescently labeled blood cells excited under epi-illumination, blood motion within the lung was captured in fluorescence emission video sequences, and the frames were spatially registered (Supporting Video 5). As opposed to the dorsal window chamber model (which employs transmitted light and uses the optical absorption of hemoglobin as the source of contrast), the pulmonary window chamber technique measures the fluorescence excitation of a small fraction of fluorescently labeled red blood cells in order to track blood movement. Despite these differences in technique, the

mapping algorithm produced speed and direction maps that correlated well with visually observed velocity features (Fig. 5a & 5b; Supporting Video 6).

Brain model

A further test of this algorithm involved the mapping of hemodynamic parameters in the pial vessels of the non-human primate brain. A small window was surgically established to facilitate optical observation of the outermost layers of intracranial tissues. The vascular network associated with the pia mater was clearly visible under epi-illumination, and movement of blood through these vessels was captured with a high frame rate camera (Supporting Video 7). Since these vessels feed directly into and out of the cortex and have been observed to modulate blood flow in response to neural stimulation, they provide an easily accessible indicator of local changes in brain activity. When electrodes were used to electrically stimulate the underlying cortex, the alterations in flow in the associated pial vessels were visually apparent.

Based on concurrent EEG measurements, the observed sequence was subdivided into periods representing baseline activity, stimulation, after-discharge, and recovery. Using the mapping algorithm, a dynamic velocity movie was generated which illustrates the vascular effects of electro-stimulation (Supporting Video 8). Velocity maps at select time points show a profound alteration in blood flow velocity beginning in the after-discharge period and rising during vasodilation of the major supplying arteriole (Fig. 6). Blood velocity in a downstream venule was also plotted using values derived from both the mapping algorithm and the dual-slit method. Velocities estimated using the mapping algorithm correlated well with dual-slit estimates ($\rho = 0.98/p < 10^{-10}$). Velocity changes estimated by both the mapping algorithm and the dual-slit method correlated with changes in the diameter of the supplying arteriole ($\rho = 0.89/p < 10^{-10}$ and $\rho = 0.86/p < 10^{-10}$, respectively). Blood velocity in the venule maintained a mean value more than twice the baseline average during the recovery period ($\bar{v}_{baseline} = 0.44 \frac{mm}{s}$, $\bar{v}_{recovery} = 1.0 \frac{mm}{s}$, $p < 10^{-10}$), suggesting that pial blood speed is sensitive to neural activity. Baseline velocities were consistent with pial blood flow data from earlier studies [27,28].

DISCUSSION

To our knowledge, no other method has demonstrated the ability to quantitatively map blood velocity with the accuracy and completeness attained by the method presented here. Within the field of view, velocities were independently derived at every pixel location, and these values were verified using the established standard for the analysis of such data. The need for human guidance is eliminated, so that entire vessel networks can be mapped quickly and without user bias. The method is applicable in a number of imaging modalities, and can be incorporated into relatively inexpensive optical systems. Thus, the mapping algorithm presented here represents a significant advance in observation of microvascular network hemodynamics.

The combination of this technique with hyperspectral imaging highlights the potential applicability of this method within a suite of imaging modalities. Combined with hemoglobin saturation images, velocity maps may also provide useful data regarding the development of vascular networks and their adaptation to inhomogeneous tissue oxygenation. These images demonstrate the feasibility of more sophisticated approaches which may provide an invaluable resource in the modeling of hemodynamics [24].

The ability to generate easily interpretable flow direction maps adds further utility to this method. Whereas many systems present magnitude data alone, the direction maps demonstrated here provide additional insight into the dynamics of complex vascular

networks. In the presented pulmonary maps, for example, the ability to visualize hemodynamic patterns provided complementary information on the functional physiology of the interrogated vasculature.

Furthermore, the automated aspect of this technique allows for the dynamic mapping of blood velocity, potentially in real time. This allows analysis of systems in which changing blood flow patterns indicate functional or physiological changes that cannot be directly observed. For example, in the dynamic mapping of blood velocity during stimulation of the cerebral cortex, the ability to generate high temporal resolution sequences allowed us to identify the episodes of after-discharge and subsequent recovery as the periods of initiation of vasodilation and largest increases in velocity, respectively. These effects are mapped and quantified here for the first time in the primate brain.

A fundamental limit to temporal resolution of dynamic blood velocity is the frame sequence over which temporal signals are cross-correlated. Here we typically used 64 or 128 frames collected at 24 or 44 Hz. This yielded temporal resolutions between 1.5 and 5.3 seconds. Over this time span, finer variations in speed are averaged out, masking blood pulsatility dynamics. This may be a positive or negative feature of the algorithm, depending on the intended application. Faster frame rates may assist in the acquisition of sub-second temporal resolutions, however, or the use of fewer frames may achieve the same goal at the expense of additional noise.

Another limitation of the method is its inability to distinguish data in three spatial dimensions. In this sense, spectroscopic methods may be superior in for certain applications in which depth information must be preserved. In a number of systems, however, vascular networks can be reasonably approximated in two spatial dimensions, or two-dimensional samples of a three-dimensional network supply representative flow characteristics. Several such examples are presented here. Prior studies have also demonstrated the ability of video-based techniques to observe and quantify microcirculatory function in a number of organs under both normal and pathological conditions [30]. Neither does this two-dimensional limitation represent a major disadvantage in the depth of vessel detection, since even the most advanced optical imaging systems are fundamentally limited to operation within a few hundred microns from the surface due to rapid photo-attenuation in tissue. And although depth sectioning cannot be directly achieved using this method, the resolvable volume (a product of the numerical aperture-determined depth of field) can be expanded with lower NA objectives, at the cost of decreased spatial resolution.

Fundamentally, the presented mapping algorithm represents a substantial advance in the processing of two-dimensional image sequences, which is a widely used method of acquiring microcirculatory hemodynamic data. In comparison to spectroscopic techniques, it holds the advantages of fast acquisition times and less in-plane insensitivity at the expense of the loss of depth information. Furthermore, its technical simplicity allows for it to be easily and inexpensively integrated into existing optical systems, making it a valuable tool in certain biological studies where the incorporation of advanced optical hardware is not practical.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Figure 1. A schematic representation of the algorithm

(1) Image sequences are recorded and saved as three-dimensional arrays. (2) For a chosen pixel, its temporal signal (blue), and the temporal signals associated with neighboring pixels are extracted (green). The temporal profiles associated with pixels within a flowing vessel are similar to the signal of interest, but with temporal offsets that depend on their upstream/downstream location (right). Signals outside the flow stream (left) fluctuate randomly and poorly correlate with the signal of interest. (3) For every signal within the local region, the temporal offset that produces the best correlation between that signal and the signal of interest is determined. Regions consisting of clusters of coordinates with common offset values are identified. These offsets increase or decrease along the flow direction. (4) By including only clusters of a certain minimum area, the flowing region is isolated from the noise. (5) The centroid of each cluster is calculated, forming a flow path from which velocity and direction can be calculated. Steps 2–5 are repeated for every pixel.

Figure 2. A Map of Frame Offsets

This image shows a map of frame offsets derived from real flow data. The temporal signal at each coordinate was compared to the signal of interest (center coordinate), and the frame offset that produced the best signal correlation (i.e. maximized the cross-correlation) was mapped. Incrementally increasing clusters of frame offsets can be seen. The flow speed calculated at this particular coordinate was 0.656 mm/s. The algorithm produces similar local correlation matrices (from which to calculate velocity) for every spatial coordinate, in turn.

Figure 3. Multi-parametric analysis of hemodynamics in 4T1 tumors grown in the skin fold window

A. Blood flow direction was derived from dorsal window chamber video recordings. The color wheel indicates the direction of motion (red represents movement to the right, blue represents movement to the lower left, etc.). B. Flow speed was mapped for values up to 1 mm/s. A number of vessels had higher speeds, but the dynamic range was limited to 0–1 mm/s in order to show differences between slower moving vessels. C. Within this same field of view, hemoglobin saturation was calculated and mapped with a color intensity weighted by total hemoglobin content. The scale bars are 500 μm.

Figure 4. Validation of the Mapping Algorithm

Within common vessel segments, velocity magnitude values were calculated from the same blood flow video using both the dual-slit method and the mapping algorithm. Data are plotted along with their linear fit. The two approaches show good agreement over a range of 0–2 mm/s for a 44 Hz frame rate and 4 μm pixel size (N=37 from two mice).

Figure 5. Direction and flow speed maps of the pulmonary vasculature

A. The direction map shows that blood emerges from pulmonary arterioles (yellow circles), traverses the lung surface and is collected by pulmonary venules (blue circles). Direction of movement is indicated by the color wheel in the top left. B. Flow speed is shown on a logarithmic scale to better highlight subtle variations in speed at lower velocities. The scale bars are 100 μm .

Figure 6. Electro-stimulation elicits a profound effect upon the pial venules in the primate brain Velocity is mapped (on a log scale) for a portion of the electro-stimulated pial network at a number of time points. At 10 and 19 seconds (representing the network prior to and during stimulation) the feeding arteriole maintains a fairly constant velocity and vessel diameter. By 30 seconds (the after-discharge period), the arteriole begins to dilate. At 45 seconds the arteriole has increased in diameter by nearly 33%, and flow velocities in the mapped network have correspondingly increased. Diameter and flow rate are plotted for the supplying arteriole (light blue arrow), along with dual-list and mapping algorithm flow rates in a down-stream venule (dark blue arrow). The gap at approximately 34 seconds corresponds to a brief period of motion artifacts. Venule velocity (as calculated by both methods) and arteriole diameter all showed statistical correlation with one another. Dual-slit arteriole velocities are not plotted due to our inability to acquire consistent data at such high flow rates. Scale bars are 100 μm .