

Simultaneous imaging of oxygen tension and blood flow in animals using a digital micromirror device

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Abstract: In this study we present a novel imaging method that combines high resolution cerebral blood flow imaging with a highly flexible map of absolute pO_2 . *In vivo* measurements of pO_2 in animals using phosphorescence quenching is a well established method, and is preferable over electrical probes which are inherently invasive and are limited to single point measurements. However, spatially resolved pO_2 measurements using phosphorescence lifetime quenching typically require expensive cameras to obtain images of pO_2 and often suffer from poor signal to noise. Our approach enables us to retain the high temporal resolution and sensitivity of single point detection of phosphorescence by using a digital micromirror device (DMD) to selectively illuminate arbitrarily shaped regions of tissue. In addition, by simultaneously using Laser Speckle Contrast Imaging (LSCI) to measure relative blood flow, we can better examine the relationship between blood flow and absolute pO_2 . We successfully used this instrument to study changes that occur during ischemic conditions in the brain with enough spatial resolution to clearly distinguish different regions. This novel instrument will provide researchers with an inexpensive and improved technique to examine multiple hemodynamic parameters simultaneously in the brain as well as other tissues.

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OCIS codes: (110.6150) Speckle imaging; (170.0110) Imaging systems; (170.3880) Medical and biological imaging; (170.6280) Spectroscopy, fluorescence and luminescence; (230.6120) Spatial light modulators.

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1. Introduction

The ability to quantitatively image oxygenation levels in tissue is important for a number of tissues and physiological conditions. In addition, pathophysiological conditions such as stroke require multiple hemodynamic parameters such as blood flow, hemoglobin saturation and volume, and oxygen tension (pO_2) to be measured simultaneously. Recent innovations in optical imaging have led to significant improvements in spatial resolution for methods that examine these parameters. Speckle contrast imaging has been shown to be a powerful method for full field imaging of blood flow [1,2], while multi-spectral reflectance imaging has improved the spatial resolution of traditional spectroscopy [3–6]. Despite these improvements, the majority of optical imaging techniques are still limited to relative measurements. This prevents a thorough analysis of the important role of baseline conditions and also fair comparisons across trials.

The one hemodynamic parameter that is typically measured in absolute values is the oxygen tension (pO_2). Clark electrodes have been the 'gold' standard for making absolute measurements of oxygen tension in tissue [7–9]. This technique has provided useful results despite being limited to point measurements and being inherently invasive. Recently, phosphorescence quenching of oxygen sensitive dyes has emerged as the standard technique for in-vivo measurement of pO_2 in animals. Phosphorescence quenching requires administration of an oxygen sensitive probe such as porphyrin based phosphors. pO_2 levels are then determined by measurement of the phosphorescence decay time which can be converted to pO_2 values using the Stern Volmer relationship. This technique has been used to effectively measure pO_2 in many different types of tissue including the brain, retina, and muscle [10–15].

Despite its widespread use, phosphorescence quenching methods are almost always limited to a single spatial location and only a few reports have demonstrated pO_2 imaging or spatial mapping [10,16,17]. These methods have typically utilized scanning lasers as an excitation source [18,19] or extremely sensitive cameras with gated exposure times [11,12,20] to achieve spatial resolution. A major drawback of these techniques is the increased time needed to either scan for multiple point measurements or perform significant averaging to improve the poor signal to noise ratios of cameras. Often for studying physiological events such as ischemia or functional brain activation, rather than have high spatial resolution, it would be useful to have just enough spatial resolution to distinguish pO_2 levels between different bulk regions or specific blood vessels.

In this paper we present a novel imaging technique to measure pO_2 and relative blood flow in arbitrarily defined regions. By using a digital micromirror device (DMD) as a spatial light modulator, variable sized regions can be excited in quick succession to create a user-defined map of oxygen tension. This approach retains the high signal to noise ratio of single point pO_2 measurements since a point detector is used rather than a camera, but also enables spatial mapping to be performed by creating spatially patterned excitation light. In addition, this approach is performed simultaneously with laser speckle contrast imaging of blood flow. We demonstrate the usefulness of this system for simultaneous measurement of blood flow and pO_2 in the brain by quantifying the effects of topically applying a vasoconstricting agent to the surface of the cortex. We also show how the DMD can be used to create ischemic regions and then analyze the pO_2 and blood flow deficits in areas around the ischemic lesion. This ability to simultaneously measure blood flow and pO_2 in user-defined regions using relatively simple instrumentation should be widely applicable to physiologic studies in the brain as well as other tissues.

2. Methods

2.1 Imaging instrument

A schematic of the imaging instrument is shown in Fig. 1(a). Laser speckle contrast imaging (LSCI) of blood flow was performed with a laser diode (808nm, 30mW) placed at a 45° angle approximately 20cm away from the animal so that the area of interest was illuminated evenly. The optical magnification used (1x and 3x) varied between animals. The scattered laser light was captured by a CMOS camera (Basler 602f, 610 x 490 pixels) at a rate of 100 frames per second and an exposure time of 5 ms using custom written software. At the same time 532nm laser light was delivered to the tissue to excite the oxygen sensitive probe. The 532nm laser was gated using an acoustic optic modulator (AOM) to produce 10 μ s pulses of excitation light at a rate of 1 kHz. It was important to use a small duty cycle (1%) because high excitation flux levels could produce damaging levels of singlet oxygen [21].

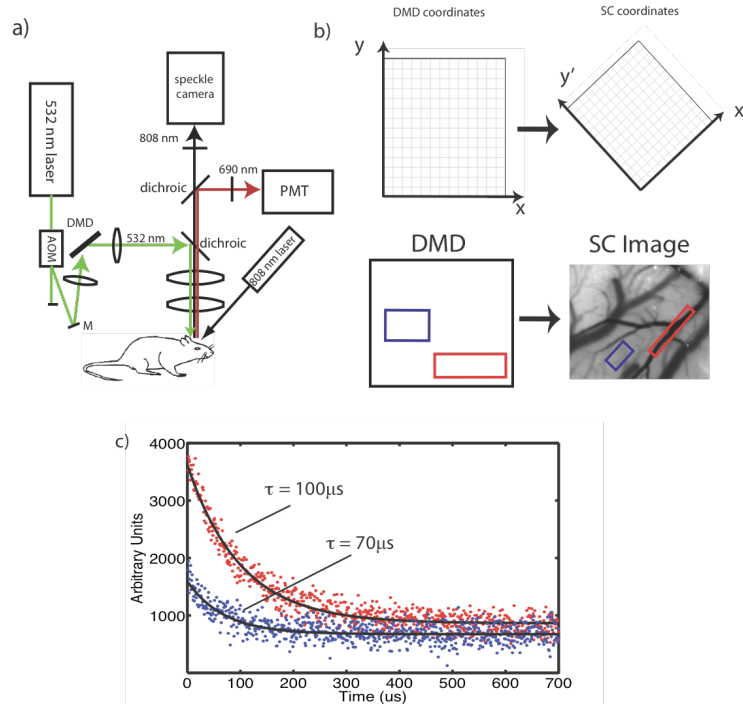


Fig. 1. (a) A schematic of the imaging setup. (b) An example transformation from DMD coordinates to the speckle contrast images. (c) Example decay curves and fitting from two regions.

The 532nm light was reflected off of a digital micromirror device (DMD) which was imaged onto the tissue surface. The DMD (Texas Instruments) was part of a Discovery 1100 kit (Digital Light Innovations) and consisted of 1024x768 individually controlled square mirrors ($\sim 13\mu m$). The DMD mirror array was coregistered with the camera using custom written software that utilized an affine image transformation (Fig. 1(b)). Arbitrary regions of interest were selected in software using the image from the camera (speckle contrast images in our case) and binary image masks were created for each region of interest and loaded onto the DMD. This allowed green excitation light to be projected onto the user-defined regions and the emitted phosphorescent light to be collected from each region sequentially (Fig. 1(c)). The phosphorescent light was separated from the speckle laser light using a dichroic mirror and collected by a photomultiplier tube (Hamamatsu R3896) after passing through two 700nm bandpass filters. The collected light represented the average phosphorescence from the entire highlighted region. The temporal resolution of the pO_2 mapping is limited by the time required to collect the phosphorescence signal over each illumination region. In our measurements, the DMD cycled through 8 regions per second and 125 phosphorescence decays were acquired for each region due to the 1 kHz repetition rate of the excitation light. When all mirrors were focused on the sample, the average power of the excitation light measured at the back of the objective was approximately 1 mW. While DMDs have previously been used for spatially resolved lifetime imaging in some microscopy applications [22–27], one has never been used for lifetime imaging *in vivo*.

2.2 Laser speckle contrast image analysis

The speckle contrast image is defined as the ratio of the standard deviation to the mean intensity in a small region of the image:

$$K = \frac{\sigma_s}{\langle I \rangle}. \quad (1)$$

A 7x7 region of pixels was used to balance the fact that the speckle contrast window must be large enough to contain a sufficient number of pixels yet not so large that significant spatial resolution is lost. Each raw speckle image was converted to a speckle contrast map in real time using highly optimized algorithms for evaluating Eq. (1) [28], and each set of 30 speckle contrast images were averaged together to increase the signal to noise ratio. This averaged speckle contrast image was then converted to an image of relative correlation times. Using the same assumptions utilized by laser-Doppler flowmetry (LDF), these correlation times were estimated from the speckle contrast values and assumed to be inversely proportional to blood flow changes [2]. Regions in these relative correlation images that corresponded to the highlighted pO₂ regions were then averaged.

2.3 pO₂ measurements

Oxyphor R2 (Oxygen Enterprises Ltd.) is a type of phosphorescent probe [Pd-meso-tetra(4-carboxyphenyl) porphyrin dendrimer] that easily dissolves in saline (28 mg/mL) and has been very effective at measuring absolute pO₂ in-vivo using phosphorescence quenching [29–31]. It has been shown to have an excitation peak near 524nm and a peak emission near 700nm [32]. The Oxyphor R2 was mixed with 90 mg/ml of bovine serum albumin (BP671-10 Fisher Scientific) to prevent leaking out of the vasculature and approximately 1 mL/kg was injected into the bloodstream of the rat via the femoral vein. The relationship between pO₂ and phosphorescence lifetime was assumed to follow the Stern-Volmer relationship

$$\frac{1}{\tau} = \frac{1}{\tau_0} + k_q[pO_2], \quad (2)$$

where τ is determined by fitting the measured phosphorescence lifetime for each region to an exponential decay (Fig. 1(c)), τ_0 is the measured lifetime in the absence of oxygen (533 μ s), and k_q is the quenching constant. The value for τ_0 was measured by sealing dissolved Oxyphor R2 in a cuvette with glucose and glucose oxidase used to consume all of the surrounding oxygen [29]. The value for k_q (356 mmHg⁻¹* μ s⁻¹) was taken from previously published studies of Oxyphor R2 [32].

2.4 Animal procedure

The Animal Care and Use Committees of the University of Texas at Austin approved all animal protocols. Three male Sprague-Dawley rats (250–350 g) were anesthetized with urethane (1.5 g/kg, i.p.). Each animal was placed in a stereotaxic frame (Kopf Instruments) and body temperature was maintained at 37 °C using a heating pad (WPI ATC1000). The femoral vein was cannulated to inject Oxyphor R2 (Oxygen Enterprises Ltd.) for pO₂ measurements. A 4mm x 4mm portion of skull on the left hemisphere around the somatosensory cortex was thinned to transparency using a dental burr and then removed. Dental cement was used to create a well around the craniotomy that was filled with silicone gel to improve visibility. In two of the animals, a second smaller craniotomy was then made lateral to the first craniotomy (Fig. 2(a)) so that endothelin-1 (ET-1), a topical vasoconstriction agent [33,34], could be applied. The location of the second craniotomy was determined by looking for a large arteriole so that we could observe the downstream effects of ET-1 on the arteriole.

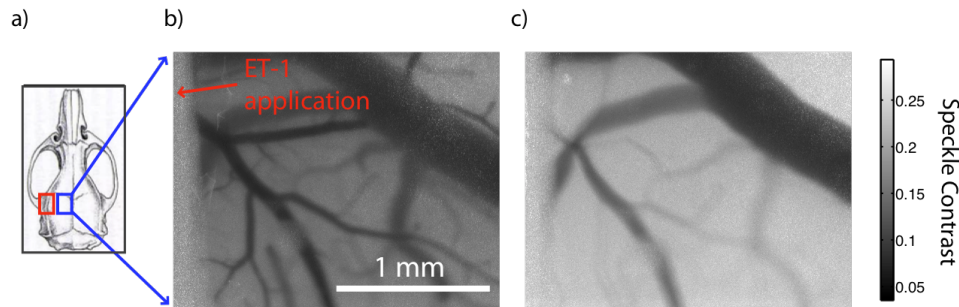


Fig. 2. (a) Location of the two craniotomies. ET-1 is applied to the lateral craniotomy and speckle contrast images are examined in the medial craniotomy (b) before and (c) 5 minutes after ET-1 application.

3. Results

We tested the ability of our system to detect changes in pO_2 and blood flow in different regions of the cortex during ischemia. To induce ischemia we topically applied ET-1, a vasoconstricting agent, to the surface of the cortex lateral to the field of view. Upon application of the ET-1 to the lateral craniotomy, the downstream blood flow decreased over the entire field of view, as illustrated by the speckle contrast images before (Fig. 2(b)) and 5 minutes after ET-1 application (Fig. 2(c)). Note that the increase in speckle contrast values is indicative of a decrease in blood flow. While there was an expected global decrease in both blood flow and pO_2 , Fig. 3 demonstrates the ability of our system to distinguish changes that occur at different regions of vasculature in a $2.3\text{mm} \times 1.7\text{mm}$ field of view. A vein, three branches of an arteriole, and four distinct regions of the parenchyma ranging in areas from 0.5mm^2 to 0.05mm^2 respectively were individually highlighted using the DMD (Fig. 3(a)). The instrument acquired 125 phosphorescence decay curves from each region resulting in a temporal resolution of approximately one second. The average decay curve in each region was fit to a single exponential decay and converted to pO_2 values using Eq. (2). The plots in Fig. 3b show the average changes in blood flow and pO_2 within each of the 8 regions.

The vein experienced the smallest decreases in relative blood flow from baseline ($\sim 10\%$ reduction) and absolute pO_2 in the vein decreased to 27 mmHg ($\sim 10\%$ reduction). While the different branches of the selected arteriole had larger decreases in relative blood flow ($\sim 80\%$ reduction) from baseline compared to the surrounding parenchyma ($\sim 70\%$ reduction), the resultant pO_2 in the parenchyma, 10 mmHg ($\sim 70\%$ reduction), was lower than the pO_2 in the arterioles, 15 mmHg ($\sim 50\%$ reduction). It is likely the arterioles had higher initial blood flow and supplied the nearby parenchyma with oxygen, so it is not surprising that its upstream constriction would lead to a severe decrease in relative blood flow and also have a significant effect on the surrounding pO_2 .

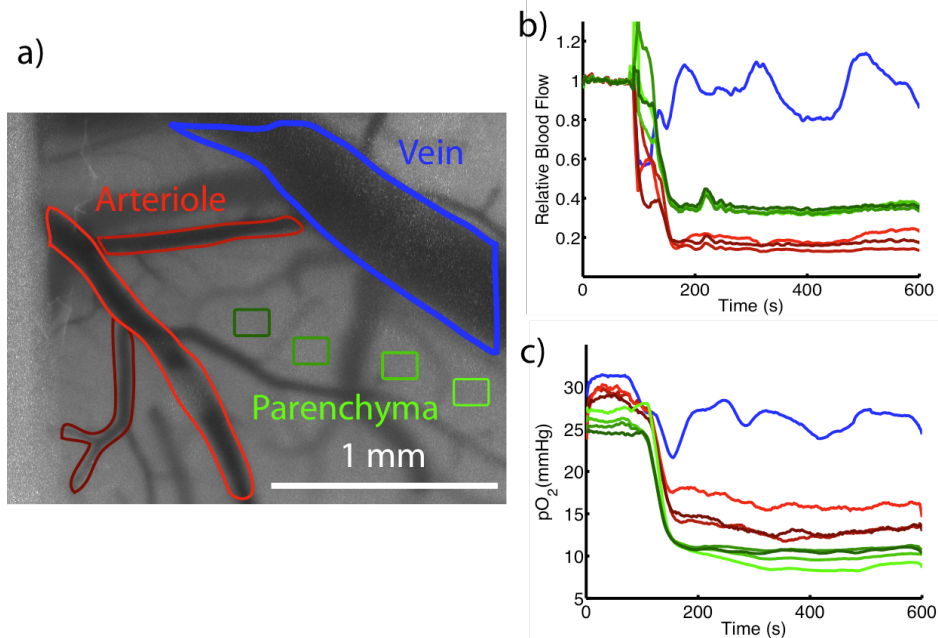


Fig. 3. (a) Various regions are selected for analysis before a topical vasoconstricting agent is applied laterally to the field of view. The speckle contrast images were then used to calculate (b) relative blood flow while the DMD is simultaneously used to measure the (c) pO_2 in the 8 regions.

In addition to distinguishing different regions of vasculature, this technique was also used with a larger field of view (7mm x 5mm) to quantify pO_2 and blood flow changes at various distances from the site of ET-1 application with a series of six rectangular regions (0.4mm x 2.5mm) as shown in Fig. 4(a). Overall the blood flow and pO_2 changes were smaller and took longer to occur in Fig. 4 compared to Fig. 3 mainly because the regions analyzed were further away from the stroke induction site. Baseline values for pO_2 were fairly uniform (29-32 mmHg) most likely due to the large size of the regions of interest. A brief drop in pO_2 ($t = 260s$) after the application of ET-1 was shortly followed by an increase in CBF ($t = 300s$) before the expected larger decrease in all parameters. After eight minutes, the closest region to the stroke induction site, about 3 mm away, experienced the largest decrease in relative blood flow from baseline (~35%) while absolute pO_2 decreased to 19 mmHg. The effects of ET-1 were less pronounced further away from the site of application, and the region that was about 6 mm away had a small decrease in blood flow (~15%) while absolute pO_2 had only dropped to 26 mmHg.

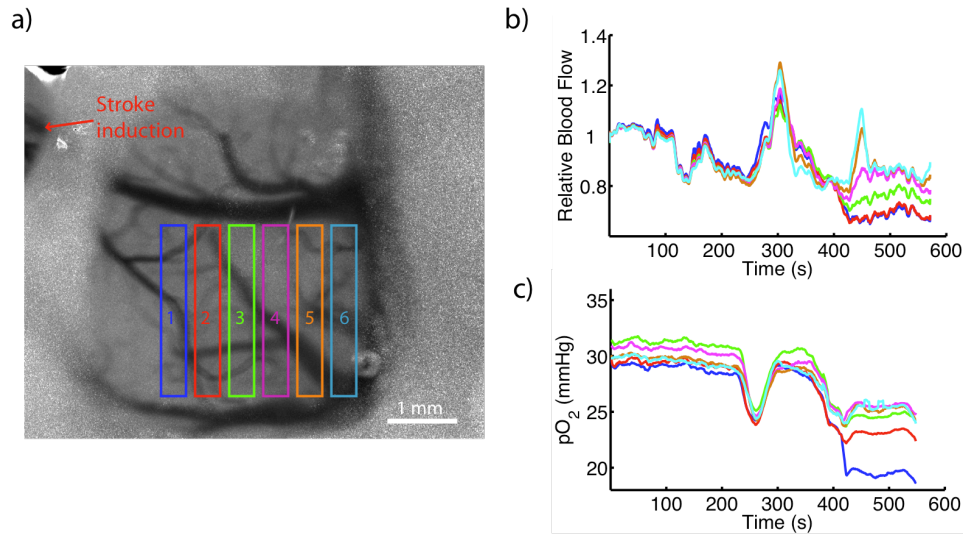


Fig. 4. (a) 6 regions are selected in a large field of view at various distances from an induced ischemia. The speckle contrast images were then used to calculate (b) relative blood flow while the DMD is simultaneously used to measure the (c) pO_2 in the regions.

Another interesting application of the DMD is to create user defined photothrombotic stroke models. Photothrombosis is a common animal stroke model where a photosensitive dye, typically rose bengal [35–37], is injected into the bloodstream. The excitation of the dye produces singlet oxygen that causes localized damage and eventually starts a clotting cascade that creates a localized ischemia. While Oxyphor R2 is not as efficient as rose bengal at producing singlet oxygen, exciting the dye with high energy fluxes has been shown to generate phototoxicity problems [21]. We created ischemic regions by increasing the duty cycle of the AOM from 1% to 25% and then using the DMD to constantly highlight one area of interest.

Figure 5 shows how the original imaging system was used to quantify pO_2 and relative CBF in four doughnut shaped regions surrounding site of photothrombosis for five minutes. The DMD was then used to induce a photothrombotic lesion in the center region while blood flow was still being measured for twelve minutes. After inducing the lesion, the system was again used to measure pO_2 in the four regions for an additional five minutes. When the DMD highlighted the center region there was an initial decrease in blood flow in the two closest regions. This was followed by a transient hyperemia before a more pronounced decrease in blood flow in all regions. The decrease in relative blood flow from baseline conditions varied across the different regions (20-60%) depending upon their proximity to the center of the photothrombotic lesion, with the closest region experiencing the largest reduction in CBF. Similarly, the initial pO_2 values in all the regions (28-32 mmHg) dropped significantly after the ischemia with lower values associated with closer regions (11 mmHg) compared to regions that were further away (16 mmHg).

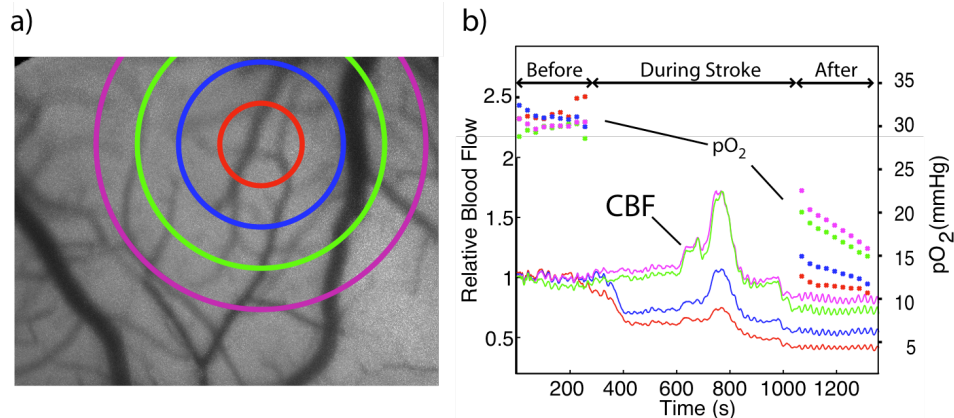


Fig. 5. (a) 4 donut shaped regions were selected while the DMD was used to create an ischemic lesion in the center of the regions through photothrombosis. (b) Relative blood flow and pO_2 were measured although pO_2 measurements could not be made while the DMD was being used to induce the stroke.

4. Discussion

Very few spatially resolved pO_2 studies have been performed in animals, primarily due to the lack of suitable methods for spatial mapping or imaging of pO_2 via phosphorescence quenching. The techniques that do exist typically involve laser scanning, which can be prohibitively slow, or gated camera based methods, which require expensive camera and heavy averaging to overcome poor signal to noise ratios. While our system also requires a camera in the beginning of the experiment in order to select regions, a relatively inexpensive camera is sufficient since the only requirement of the camera is adequate visualization of the tissue surface. Furthermore, it is straightforward to also use this camera to perform speckle contrast imaging, and therefore measure spatially resolved pO_2 and blood flow simultaneously. By moving the spatial analysis of the phosphorescence lifetime measurements to the illumination component, our technique utilizes the high sensitivity and speed of point detectors while retaining enough spatial resolution for distinct regional analysis. In this system the spatial and temporal resolution are both essentially limited by the signal to noise ratio. We were able to reliably excite regions as small as 0.05mm^2 with a temporal resolution of eight regions per second (due to the averaging of 125 phosphorescence decay curves at a repetition rate of 1 kHz) and could potentially improve either if needed. While we use an expensive laser as an excitation source because of its availability in our laboratory, the entire system could become easier to implement and considerably cheaper using powerful LEDs [13]. Additionally, the concept of using a DMD for regional selection does not have to be limited to oxygen measurements, and it could be useful for analyzing numerous fluorescent probes that are traditionally difficult to image because of poor signal to noise ratios.

The application of ET-1 in small amounts has been shown to induce spreading depressions [38,39] which are commonly associated with a temporary increase in blood flow [40,41]. These events have been shown to be preceded by a dip in pO_2 [20] much like the event seen in Fig. 4(c). Similar spreading depolarizations are commonly seen in the penumbra region of an ischemic stroke [42–44]. These peri-infarct depolarizations (PIDs) are also associated with a wave of increased blood flow despite the overall decrease in blood flow from the ischemia. The waves of increased blood flow seen in Figs. 4(b) and 5(b) seem consistent with some form of spreading depolarization and again highlight the need to closely monitor multiple parameters during stroke.

One consideration when selecting individual regions for pO_2 measurements is that light could scatter outside the region of interest. This is especially concerning when selecting small individual vessels, but is essentially the same problem faced by many optical based techniques

including laser speckle contrast imaging and intrinsic optical imaging. This could help explain why the oxygen tension in the arterioles is not as high as expected since light could be scattering outside the vessel and into the less oxygenated parenchyma. Anesthesia also plays an important role in baseline oxygen conditions which could also help explain the low pO_2 values. We have found that pO_2 values in the parenchyma of urethane anesthetized animals (27 ± 2 mmHg), like the ones used in these experiments, are significantly lower than isoflurane anesthetized animals (46 ± 3 mmHg), similar to what other groups have seen [45]. Another concern is that since the scattering of light is wavelength dependent, the speckle and pO_2 measurements may sample different tissue depths. This issue has been addressed by many groups that combine LDF or LSCI with spectroscopic reflectance measurements [4,42,46,47], and as been found to be insignificant when studying events like functional activation [3]. One could assume it would be even less significant for studying more global events like stroke.

5. Conclusion

We have presented a novel imaging technique that uses a digital micromirror device to provide highly flexible maps of pO_2 in animals while simultaneously imaging relative blood flow using LSCI. This technique provides better temporal resolution than laser scanning or more expensive camera based methods while retaining enough spatial resolution for distinct regional analysis. We demonstrated the ability of this system to successfully monitor decreases in pO_2 and blood flow during ischemic events in the brain by applying topical vasoconstriction agents to the rat cortex or using the DMD to induce photothrombosis. Being able to monitor these physiological changes is extremely important for understanding ischemic events and quantifying the efficacy of different treatments.

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