Laser speckle analysis of retinal vascular dynamics

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Abstract: Studies of vascular responses are usually performed on isolated vessels or on single vessels in vivo. This allows for precise measurements of diameter or blood flow. However, dynamical responses of the whole microvascular network are difficult to access experimentally. We suggest to use full-field laser speckle imaging to evaluate vascular responses of the retinal network. Image segmentation and vessel recognition algorithms together with response mapping allow us to analyze diameter changes and blood flow responses in the intact retinal network upon systemic administration of the vasoconstrictor angiotensin II, the vasodilator acetylcholine or on the changing level of anesthesia in in vivo rat preparations.

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OCIS codes: (120.6150) Speckle imaging; (170.1470) Blood or tissue constituent monitoring; (170.5380) Physiology.

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1. Introduction

The retina has a highly fluctuating metabolic demand: an increase in incident light immediately increases the local metabolism necessitating a parallel increase in blood flow [1]. The microcirculation supplying this specialized tissue therefore has a pronounced ability to regulate flow in order to satisfy local metabolic demand at any time. Insufficient supply, but also overperfusion [2] potentially leads to tissue damage and visual impairment. Unlike other microvascular beds, the retinal microcirculation can be easily accessed \textit{in vivo} without disturbing the delicate structure and environment of the network. Besides, a number of systemic conditions such as hypertension and diabetes [3, 4] cause vascular manifestations in the eye. Monitoring of retinal vascular network can be useful for disease staging and for evaluating disease progression. Taken together the retina therefore becomes a very interesting tissue for studying microvascular flow under both normal and pathologic conditions.

Laser speckle imaging (LSI) has been successfully applied to many biomedical systems [5–7]. It has a distinct advantage over other techniques for monitoring of blood flow as it is non-scanning, full-field technique with high temporal and spatial resolution. Since the first studies in retina [8], different aspects of retinal blood flow and ophthalmological problems have been addressed. Srienc et al. [9] focused on the fundamental question of how blood flow is regulated at the local level in the retina. Flammer et al. [10] employed the method to investigate the effects of glaucoma on retinal circulation whereas Watanabe et al. [11] imaged choroidal hemodynamics in patients with polypoidal choroidal vasculopathy. An overview of the use of laser speckle imaging in ocular fluid flow research is given in Ref. [12]. Recently, in tissues other than the retina, much efforts have been made to evaluate dynamical patterns of blood flow oscillations using LSI [13–17].

In the present paper we evaluate the dynamical response of the retinal vascular network following a change in systemic pressure. The goal is to develop a method for studying dynamical flow changes in the whole network, rather than confining the measurements to a single vessel. Hence, the flow pattern is measured simultaneously in a large number of vessels. More specifically we evaluate relative changes in blood flow velocity and estimate blood flow upon systemic administration of vasoactive agents (angiotensin II and acetylcholine) or changes in the level of anesthesia (isoflurane).
2. Methods
2.1. Experiment

All experiments were performed on male Sprague-Dawley rats, body weight 270-330 g, purchased from Taconic (Lille Skensved, Denmark). The experimental protocol was approved by the Danish National Animal Experiments Inspectorate and were conducted in accordance with guidelines of the American Physiological Society. An IR Laser Module (LDM785, 785 nm, 20 mW, Thorlabs, Newton, New Jersey) was used as a laser source. An endoscope (5 mm of diameter, 11.5 cm of length, Karl Storz 67260AA, Tuttingen, Germany) delivered laser light from the source to the rat eye [18]. A CMOS Camera (acA1300-60gmNIR, Basler, Ahrensburg, Germany (25 frames per second, 800 × 800 pixels, 5 ms of exposure time) recorded the signal.

Before surgery anesthesia was induced by 5% isoflurane delivered in the mixture of 35%O₂ and 65%N₂. During the surgery isoflurane concentration was reduced to 2%. Two polyethylene catheters were placed in the left jugular vein (PP-10) for infusion and one catheter was placed in the right carotid artery (PP-25) and connected to a pressure transducer (Statham P23-dB, Gould, Oxnard, CA) for continuous measurement of the systemic blood pressure. After a tracheotomy and insertion of a tube into the trachea, the rat was placed on a servo-controlled heating table to maintain body temperature at 37°C. The rat was ventilated by a small-animal ventilator (tidal volume 1.7–2.3 ml, 60 breaths/min). The final isoflurane concentration needed to maintain sufficient anesthesia was ≈ 1.5%. An intravenous (I.V.) bolus injection of the muscle relaxant gallamine (Sigma G8134) of 6.6 mg/kg in 0.4 ml 0.9% saline was hereafter given followed by continuous intravenous infusion of gallamine (0.33 mg/kg) in saline (20 μl/min) to paralyze the rat and prevent eye movements. Subsequently atropine (1% solution) was applied on the corneal surface to dilate the pupil.

Experimental protocol included control period followed by systemic (intravenous) bolus injections of AngII in different concentrations, continuous intravenous infusion of ACh, and changing level of anesthesia.

![Fig. 1. Representation of the experimental set up (left panel) and the experimental protocol (right panel). Experimental setup includes endoscope (1), laser module (2), and CMOS camera (3). The rat is anesthetized and paralyzed. Experimental protocol includes systemic (intravenous) bolus injections of AngII in different concentrations, continuous intravenous infusion of ACh, and changing level of anesthesia.](image-url)
cause any changes in retinal blood flow.

To obtain a scaling factor of laser speckle images we focused on the 40 μm thread and estimated the micron-per-pixel relation.

2.2. Analysis

Recorded raw data were processed using temporal laser speckle contrast analysis. Speckle velocity (SV) was calculated as in Ref. [20]:

\[ SV = \frac{\bar{I}^2}{\sigma^2}. \]

Here \( \bar{I} \) is the mean light intensity and \( \sigma \) is its standard deviation calculated over 25 frames for each pixel. \( SV \) is proportional to the mean velocity of the scattering red blood cells in the vessel lumen. The resulting laser speckle data are composed of frames of 800 × 800 pixels each with a sampling time of 1 s. Images were acquired continuously during the observation time of 75 min. To remove small movements in the XY-plane all frames were processed through an automated intensity-based image registration with translational and rotation transformations applied to each frame.

For further analysis we developed an algorithm of image segmentation and vessel recognition:

- Each LSI frame was divided onto 100 regions of the same size (80 × 80 pixels, in our case);
- Mean SV frame was calculated over the observation time of 75 min and used for rough estimation of a vessel position in each region;
- To reduce noise in the speckle data, each frame was processed through a Gaussian smoothing algorithm with standard deviation over a region of 5 × 5 pixels and through a moving average over 3 s that corresponds to 3 subsequently acquired frames;
- A precise vessel position was identified for each frame by tracking the SV decrease from the local SV maximum in both X and Y directions;
- Typically, several vessels could be identified for each region. The largest vessel was chosen automatically for further analysis and a dynamical mask for the chosen vessels was calculated in all regions. Figure 2 shows an averaged mask over all frames.

A dynamical mask marks pixels belonging to the chosen vessel on each frame. Note that the same pixels can be inside or outside the vessel on different frames because of movements or diameter alterations. Using dynamical masks we extracted information about mean SV values and vessel diameter. To detect dynamical changes, mean values were calculated along the center line of each vessel for all frames. All segments belonging to the same vessel were marked manually and data were combined with weight coefficients:

\[ \langle SV \rangle_{line} = \sum_i \left( \langle SV \rangle_{line_i} \times \frac{N_i}{N_{total}} \right), \]

where \( \langle SV \rangle_{line_i} \) is an element of an array of SV values along the center line of the current segment. \( N_i \) is the number of pixels in the center line of the current segment. \( N_{total} \) is the total number of pixels in the center lines of all segments related to the vessel. Index \( i \) in the sum is running over the number of segments of a certain vessel. Vessel diameter was estimated according to the algorithm described in Ref. [21].
Fig. 2. Example of vessel detection based on $SV$ data segmentation. Left panel: Mean laser speckle image. Right panel: The result of vessel detection. Purple color indicates selected vessels.

To calculate a velocity response map a mean value $SV_R$ calculated over the peak response period ($R$ in Fig 3c) is divided by a mean value $SV_B$ calculated over the baseline period ($B$ in Fig 3c). The resulting map $V_{map}$ shows changes of relative velocity in each vessel in response to stimulation:

$$V_{map} = \frac{(SV)_R}{(SV)_B}, \text{ where } (SV)_T = \frac{1}{T} \sum_{t}^T (SV)_t, \ T = R, B,$$

(3)

where $T$ is the number of frames acquired during periods $R$ and $B$ while $t$ is a frame index. In our case, $T$ is 150 frames corresponding to 150 s.

To calculate a flow response map $F_{map}$ the mean response value $SV_R$ calculated over period $R$ was multiplied by the square of the mean diameter $D_R$ over the same period and then divided by a mean value $SV_B$ calculated over the baseline period:

$$F_{map} = \frac{(SV)_R \times D_R^2}{(SV)_B \times D_B^2}.$$  

(4)

Since we calculate relative flow changes we omitted constant coefficients.

3. Network vascular response

The segmentation and vessel detection algorithm was applied to LSI data from 5 rats and gave information about 106 arterial and venous vessels, 17 branching points with about 40 interconnected vessels. A mismatch between the number of branching points and interconnected vessels reflects the fact that not all daughter vessels could be identified during segmentation. The largest vessel had a diameter of 188 $\mu$m and the smallest had a diameter of 33 $\mu$m.

A typical response of a single vessel is shown in Fig 3b: (i) $SV$ increases after the first bolus injection of AngII (2 ng); (ii) $SV$ response is stronger after the increased dose of AngII (4 ng); (iii) there is a two-phase response with decreasing $SV$ followed by an increase during ACh infusion and (iv) $SV$ drops significantly after increasing concentration of isoflurane. As shown in Fig 3 these changes mirror quite well the changes in the mean arterial pressure (MAP) whereas a relation to local vessel diameter is not obvious in all cases.
Fig. 3. Vascular responses of a retinal network. (a) Intra-arterial blood pressure. Circles refer to time points for a given intervention: Red circles indicate bolus injections of AngII of 2 ng and 4 ng. Blue circles indicate the start and the end of ACh infusion. Green circles correspond to the start and the end of increased concentration of isoflurane. (b) SV response of the two vessels V1 and V2 marked in (d) and (e) panels. (c) Diameter changes in V1 and V2 vessels. The diameter values were smoothed by using a moving average filter. (d) Delay map of blood flow velocity marks a delay of vessel response to the first AngII injection. (e) Duration map of blood flow velocity marks a duration (width at half-height) of vessel response to the first AngII injection. (f) Speckle velocity response map to Ach infusion. SV values are normalized by baseline values hence a value of 1 corresponds to no change in SV. (g) Normalized blood flow response map to Ach infusion. Color codes the strength of the response.
Mapping makes possible monitoring of both strength and dynamical feature of the response. A delay map can be used to determine which vessels of the network react first to the drug administration. In the case of systemic drug administration (AngII injection), the delay difference in the response can be caused by vascular structure where daughter vessels demonstrate delayed response compared to parent vessels (Fig. 3(d)). A duration map (Fig. 3(e)) introduces another measure of dynamical response that can distinguish vessels with different properties.

Figure 3(f) shows the $SV$ network response map following acetylcholine infusion. $SV$ values are averaged over period $R$ during ACh infusion and normalized by $SV$ values averaged over period $B$ before the infusion according to Eq. (3). The figure depicts how $SV$ responses are unevenly distributed along the network without obvious correlation to vessel diameter or vessel position. Since we recorded simultaneously $SV$ and vessel diameter, it is possible to estimate blood flow changes in the network (Fig. 3(g)) according to Eq. (4).

![Fig. 4. Reaction of 106 vessels to different stimuli. (a) Mean arterial pressure (MAP) and (b) maximal response of $SV$ normalized by the baseline value for a bolus injection of AngII (2 ng), for a bolus injection of AngII (4 ng), at the first phase of ACh infusion (P1), at the second phase of ACh infusion (P2), and during increasing level of anesthesia. Different colors indicate different animals. Each symbol corresponds to an individual vessel. Dashed line indicates the level without changes.](image)

Below we analyse $SV$ dynamical responses. Figure 4 demonstrates the relative change in MAP and $SV$ in all the observed vessels (one symbol for each vessel) for 5 different animals (marked in different colors). Statistics of vascular responses normalized by the baseline values before stimulations is summarized in Fig 4. As seen from the figure the reaction to AngII is generally stronger for the larger bolus, likely to reflect primarily the larger increase in MAP. Along the same line all vessels show a small decline in $SV$ and a subsequent rise following ACh infusion.
and, additionally, a large decline in $SV$ following an increase in isoflurane. Taken together therefore the patterns shown in Fig. 3(a),(b) are consistent and are found in all the observed vessels. As shown in Fig. 4, we generally find a quite pronounced change in $SV$ following a given intervention, however as also evident from the figure, there is a substantial variation in the data, e.g. in 16 vessels out of 106 the reaction to the second injection of AngII was weaker compared to the first injection. Also in 6 out 106 vessels, $SV$ did not show any significant reaction on AngII injection.

How is the strength of vascular responses related to the vessel diameter at resting state (i.e., without stimulus)? Subsequently, as shown in Fig. 5, we calculated the correlation between the change in $SV$ following stimulation and the baseline vessel diameter. Generally this correlation is relatively weak being largest following AngII stimulation. In that case the correlation is negative, i.e. small vessel tend to have a relatively stronger change in $SV$ as compared to larger vessels. In addition to the correlation coefficients calculated for the two different concentrations of AngII we included an additional relation: the strength of the response to 4 ng AngII relatively to the response to 2 ng AngII (third group of bars). The data show the same tendency. For stimulation with ACh or isoflurane the correlation with baseline diameter is too weak to allow for interpretation (data are not shown).

The present setup where data are recorded from all parts of the network simultaneously allows for the tracking of specific changes through the network, e.g. how $SV$ in parent and daughter vessels are related across a branch point. Fig. 6 shows the two AngII stimulations and the ACh infusion similar to that shown in Fig. 3. As expected parent and daughter vessels typically shows a relative change $SV$ in the same direction (positive or negative), e.g. Fig. 6(a), but not necessarily to the same degree (b-d).

4. Conclusion

We applied LSI to study blood flow dynamics of the intact vascular network at the level of the individual vessel response:
Fig. 6. Examples of parent-daughter responses. (a) Parent vessel and its daughter vessels demonstrate almost the same dynamics; (b) Parent vessel demonstrates much stronger reaction on the second injection of AngII and on ACh infusion; (c) the first daughter vessel shows weaker reaction on the second AngII injection but stronger reaction on ACh infusion, while the second daughter vessel demonstrates opposite dynamics similar to the parent vessel whose response is weaker, however; (d) the first daughter vessel demonstrates reaction on both AngII injections, while the second daughter vessel does not show an increase of SV on the second injection.

- Bolus injection of AngII leads to an acute increase in MAP [19] following general vasoconstriction. Increased MAP will lead to increasing erythrocyte velocity also if vessel diameter locally in the retina remains unaffected. Our measurements are in accordance with this;

- Administration of ACh is expected to cause a decrease of heart rate and MAP and, locally in the microcirculation, cause vasodilation. Consequently, we would expect to see decreasing SV. The ACh-induced response in the present case appear to involve a combination of different mechanisms since almost all vessels demonstrated two-phase response: an initial reduction in SV that correlated with a drop in MAP [22], followed by a slow increase in SV that starts during the infusion or right after and lasts for 6-7 min. This phase is probably caused by a complex systemic reaction and is associated with a slow return of the MAP towards the baseline value;

- Isoflurane like many other anesthetics causes vasorelaxation. A rapid increase in isoflurane concentration therefore decreases MAP and as a consequence leads to a fast drop of SV.
In the agreement with other experimental observations [23,24], analysis of network dynamics on different systemic stimuli revealed a tendency for smaller vessels to react stronger to a bolus injection of AngII as compared to larger vessels. Such analysis is possible due to an automatic SV and diameter estimation.

Our approach allows to map relative changes in the entire network. In addition to the mapping of the relative strength of the response, it is possible to monitor such dynamical features of the response as duration and delay. Such mapping opens opportunities to distinguish arteries and veins or to detect local pathology but requires further modifications and improvements.

Laser speckle imaging makes it possible to follow reaction of vessels connected via branch point (parent-daughter-daughter vessels). While in most cases interconnected vessels showed similar dynamics, specific response patterns were detected as well. This indicates the need to study vessels within the network environment rather than in isolated preparations.

Acknowledgments

The work was made possible by a Marie Curie grant from the European Commission in the framework of the REVAMMAD ITN (Initial Training Research network), Project number 316990.