# Characterization of the spatiotemporal responses of surface and penetrating cerebral arterioles using two-photon microscopy


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## Introduction

A mechanistic understanding of the hemodynamic response requires a systematic analysis of single-vessel properties carried out in the framework of realistic vascular microarchitecture and with respect to the underlying neuronal activity. Previously, we used somatosensory stimulation and two-photon microscopy to study neurovascular coupling in rat primary somatosensory cortex [1]. Our results showed that on the level of single surface (pial) arterioles, the response was composed of dilatory and constrictive phases. Comparison of two-photon data with neuronal measurements demonstrated a correlation of the arteriolar constriction with enhanced neuronal inhibition. Here we extend the two-photon measurements across cortical depth and to the other two vascular compartments, capillaries and veins.

Pial arterioles are influenced by propagating ("upstream") vasodilation and vasoconstriction [2], and receive sympathetic, parasympathetic and trigeminal innervation [3]. Thus, in principle, their behavior can differ from that of the pre-capillary arterioles and capillaries that are influenced directly by release of mediators from the surrounding tissue. In addition, pial arterioles are interconnected by a network of anastomoses and are expected to be less specific to the underlying cortical columns than penetrating arterioles that feed well-defined territories [4]. Capillaries have been shown to increase their diameter in response to hypercapnia and steady-state stimulation *in vivo* [5-7], and to constrict in response to direct stimulation of pericytes *in vitro* [8]. Veins have been postulated to passively increase their diameter in a baloon-like fashion, and their dilation has been hypothesized to underlie BOLD fMRI contrast [9-10]. However, direct experimental characterization of vascular stimulus-evoked responses across cortical depth and vessel-types is missing.

Here we measure changes in diameter and blood flow velocity of individual arterioles, capillaries and venules down to 500 µm in response to a somatosensory stimulus in rat primary somatosensory cortex (SI). We ask the following questions: (1) do penetrating arterioles and their branches show biphasic response similar to that of the pial arterioles? (2) will the arteriolar response depend on its cortical depth and its local vascular structure? (3) are there "hot spots" of arteriolar dilation consistent with anatomical literature [11-13]? (4) can capillaries dilate and constrict *in vivo* in response to our stimulus? (5) do veins/venules dilate in response to the stimulus? (6) is the velocity profile consistent with the diameter change?

## Methods

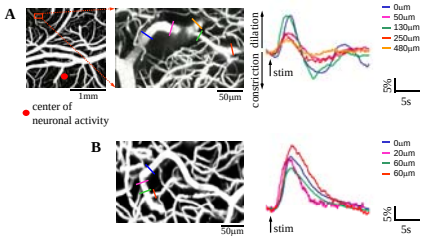
Rats were initially anesthetized with isofluorane (3% initially, 1-2% during ventilation) and ventilated with a mixture of air and oxygen during surgical procedures. During the surgery cannulas were inserted in the femoral artery and vein. Isofluorane was discontinued, and anesthesia was maintained with 50 mg*kg-1 intravenous bolus of α-chloralose followed by continuous intravenous infusion at 40 mg*kg-1*h-1. Heart rate, blood pressure and body temperature were continuously monitored. Respiration was aimed to maintain $PaCO_2$ between 35 and 45 mmHg.

An area of skull overlying the primary somatosensory cortex was exposed and then thinned until soft and transparent. A well of dental acrilic was build and filled with saline. A chamber consisting of a metal frame and a removable glass coverslip lid was glued to the skull. The thinned skull and dura mater were removed. The space between the exposed brain surface and the coverglass was filled with 0.7 % (w/v) agarose (Sigma) in ACSF. To avoid herniation of the exposed brain due to excessive intracranial pressure, dura mater over the IVth cerebral ventricle was punctured and plastic PE50 tube was inserted to allow draining of cerebro-spinal fluid (CSF). The draining hole was sealed after sealing of the imaging well.

To visualize the vasculature, ~0.3 ml of 5 % (w/v) solution of 2 MDa fluorescein-conjugated dextran (FD-2000S, Sigma) in physiological saline was injected intravenously [4]. The dye labeled blood plasma, while blood cells looked like dark shadows on the bright background.

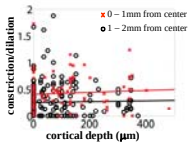
Images were obtained using Ultima 2-photon microscopy system from Prairie Technologies. We used a 4x air objective (Olympus XLFluor4x/340, NA=0.28) to obtain images of the surface vasculature across the entire cranial window to aid in navigating around the cortical vasculature. 10x (Zeiss Achroplan, NA = 0.3) and 20x (Olympus, NA=0.5) water-immersion objectives were used for high-resolution imaging and line scan measurements. Data analysis was performed in Matlab environment.

## Stimulus-evoked diameter changes of penetrating arteriole and its branches depend on distance from the center of neuronal activity




**A.** Maximal intensity projection (MIP) of low magnification image stack (4x) and MIP of high-resolution image stack (20x, 0-300 µm) of the region of interest (ROI). The location of the ROI is marked on 4x map by a red rectangle. The MIP of the ROI shows vascular structure of the measured surface, penetrating arteriole and its branches. Color-coded lines correspond to time-courses of diameter change shown on the right. Note that the biphasic response is evident at all measured depths

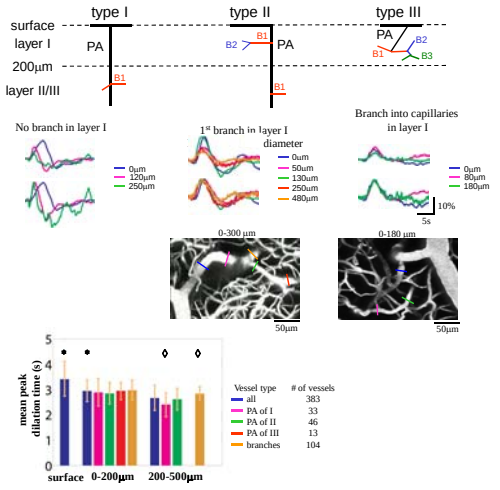
**B.** Another example close to the center of neuronal response. MIP at 20x corresponds to 0-100 µm.



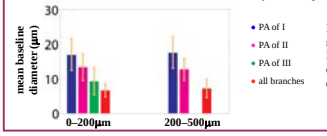

1. Diameter change of penetrating arterioles (PA) and their branches largely mimic that of the parent surface artery
2. Ratio of peak constriction/peak dilation is mostly conserved as a function of depth.
3. Ratio of peak constriction/peak dilation changes as a function of distance from the center of evoked neuronal activity: relatively to the initial dilation there is more constriction in the surround. This is consistent with our previous observations regarding surface arterioles [1].

## Stimulus-evoked diameter changes of penetrating arterioles and its branches depend on cortical depth and vascular topology

3 types of topology of penetrating arteriole (PA) and branches (B1, B2, B3)

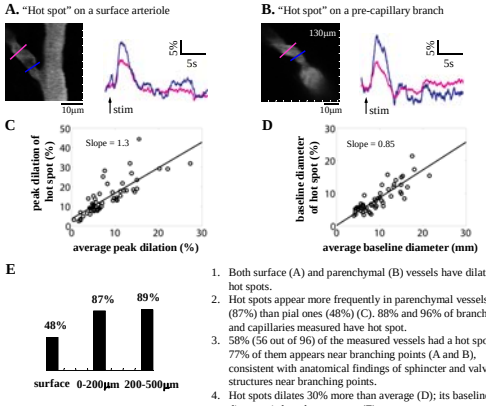



1. Dilation onset of parent pial arterioles, defined as 3 std of the baseline variance, was delayed in comparison with PA and their branches in layer I (1.1s versus 0.9s; p-value $9x10^{-4}$) (data not shown). Likewise, pial arterioles reach dilation peak later than PA and their branches (3.4s versus 3s; p-value < $1x10^{-4}$). This is consistent with the mechanism of upstream propagation.
2. PA (Type I) in layer II/III reach dilation peak earlier than pre-capillary branches (2.4s versus 2.8 s; p-value<$5x10^{-4}$).
3. There is no statistical difference in the dilation onset (but not time to peak) throughout the measured depth for PA, their branches and capillaries. This is consistent with the fact that propagation of neuronal activity throughout and across the columns is fast on hemodynamic response time scale.



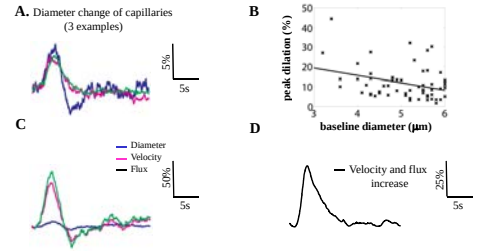

In our data, PA of type I has the largest mean baseline diameter (17mm), while PA of type III has a baseline diameter of 9.3mm, consistent with their definition.

## Dilation "hot spots": stimulus-evoked diameter change at branching locations are larger than the average diameter change



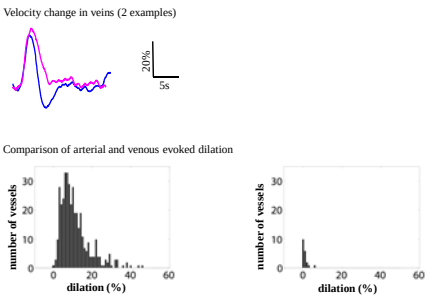

1. Both surface (A) and parenchymal (B) vessels have dilation hot spots.
2. Hot spots appear more frequently in parenchymal vessels (87%) than pial ones (48%) (C). 88% and 96% of branches and capillaries measured have hot spot.
3. 58% (56 out of 96) of the measured vessels had a hot spot; 77% of them appears near branching points (A and B), consistent with anatomical findings of sphincter and valve structures near branching points.
4. Hot spots dilates 30% more than average (D); its baseline diameter is less than average (E).

## Capillaries can both dilate and constrict




**A.** 61 capillaries (< 6 µm in our study with mean diameter = 5.1±0.7µm) show stimulus-evoked diameter change, similar to surface and parenchymal arterioles. 23 out of 24 (96%) measured capillaries have hot spots.
**B.** Smaller capillaries dilate more, consistent with findings from hypercapnia study and stead-state functional stimulus.
**C.** Example of biphasic velocity change (increase followed by decrease), leading to a biphasic flux change.
**D.** Example of velocity increase without diameter change, thus a flux increase.

## Veins do not dilate, venous velocity can be biphasic reflecting arteriolar biphasic diameter change

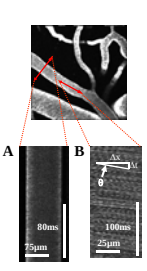



1. The majority of veins do not dilate/constrict in response to our stimulus.
2. Mean velocity increase: 50%±38%, thus its flux change is dominated by velocity change.
3. Mean peak time of velocity increase: 2.8±0.4s, similar to that of diameter change in arterioles and capillaries, thus velocity change of veins have similar profile as that of the diameter change in arterioles.

## Conclusions

1. **Penetrating arterioles and their branches (parenchymal arterioles) can have a biphasic stimulus evoked response similar to their parent surface arteries: initial dilation followed by constriction. Similar to surface arterioles, penetrating arterioles and their branches show more constriction further away from the center of neuronal activity.**
2. **The response of surface arterioles is delayed relative to parenchymal arterioles and capillaries. This is consistent with the upstream propagation of vasomotion.**
3. **Timing differences of surface and penetrating arterioles can be explained by individual geometries of vascular trees.**
4. **Parenchymal vessels show no statistical difference in the onset time of vasodilation throughout the measured depth and across different vessel types. This is consistent with the fact that propagation of neuronal activity throughout and across the columns is fast on hemodynamic response time scale. This may also imply that parenchymal vessels receive the same trigger signal for dilation onset.**
5. **Larger diameter penetrating arterioles that do not branch in layer I reach dilation peak earlier than pre-capillary branches in layer II/III.**
6. **Dilation "hot spots" are mainly located near arteriolar branching points, both on the surface and throughout the measured depth. Most capillaries also have "hot spots" that might reflect position of pericytes. Branching penetrating arterioles have are more hot spots than surface arterioles.**
7. **Capillaries can also exhibit a biphasic stimulus-evoked diameter change, indicating active diameter control.**
8. **The profiles of blood flow velocity of arterioles and capillaries are consistent with their diameter changes, resulting in a decrease in flux during vasoconstriction.**
9. **Veins do not show dilation but increase the flow velocity. Their velocity change can be biphasic. Thus, venous velocity can decrease during arteriolar constriction.**

## Estimation of diameter/velocity from line scan




$$velocity = \frac{\Delta x}{\Delta t}$$

$$\tan\theta = \frac{\Delta t}{\Delta x}$$

$$velocity = \frac{1}{\tan\theta}$$

***Diameter estimation*** – To estimate the diameter change, we obtain a reference profile. By comparing this reference to subsequent data, we can estimate how much the vessel edges has shifted and using this information, we can calculate the diameter change. The method can be used to analyze data obtained from line scans performed across the vessel (A), as well as data from freedraw line scans. The only requirement is that the line must pass through both edges of the vessel.

***Velocity estimation*** – Line scans are performed along the axis of the vessel (B). When the lines are displayed together, they form a space-time image. The dark streaks are formed by the movement of the non-fluorescent red blood cells. The velocity can be estimated from estimating the slope of the dark streaks (see equations below).

## Estimation of diameter from frame scan

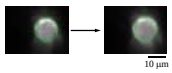
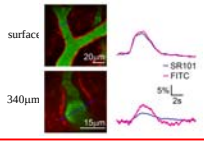



Image time series of diving vessel are obtained using frame scanning. The area/diameter change of the vessel is estimated by counting the number of pixels above an intensity threshold
Left: 2-D image of vessel at 1s before stimulus, with green reference circle
Right: the same vessel at 3s after stimulus onset

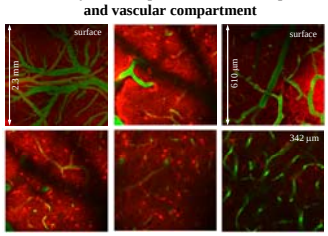
## Estimation of diameter from movement of astrocytic endfoot

Using both SR101 and intravascular fluorescein-conjugated dextran, we compared diameter change estimated directly from vessel dilation and from movement of the astrocytic endfoot. SR101 shown in red, fluorescein in green. Color-coded corresponding estimated diameter changes are shown on the right.




1. For surface arterioles diameter estimation using SR101 and fluorescein yield similar results (top example).
2. Dilation of PA can be observed with SR101 with smaller %change.
3. Constriction of PA is not always observed with SR101 (bottom example).

## Astrocytic envelop as a function of depth and vascular compartment




1. Pial arteries are outlined by a double-line with SR101.
2. Pial veins have no SR101 outline.
3. Diving arterioles and venules are surrounded by astrocytic endfeet.
4. Capillaries down to 300 µm are surrounded by astrocytic endfeet.
5. Capillares deeper than 300 µm do not show resolvable astrocytic envelop. This might be related to weaker staining of deep astrocytes using surface SR101 loading.

## References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