Journal of Visualized Experiments

In Vivo Three-Dimensional Two-Photon Microscopy to Study Conducted Vascular Responses by Local ATP Ejection Using a Glass Micro-Pipette

--Manuscript Draft--

Article Type:	Invited Methods Article - JoVE Produced Video
Manuscript Number:	JoVE59286R2
Full Title:	In Vivo Three-Dimensional Two-Photon Microscopy to Study Conducted Vascular Responses by Local ATP Ejection Using a Glass Micro-Pipette
Keywords:	in vivo, two-photon microscopy, cerebral blood flow (CBF), astrocytes, three dimensions (3D), neurovascular coupling, calcium imaging, viral vector injection
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Additional Information:	
Question	**Response**
Please indicate whether this article will be Standard Access or Open Access.	Standard Access (US$2,400)
Please indicate the **city, state/province, and country** where this article will be **filmed**. Please do not use abbreviations.	Copenhagen, Denmark

Dear Sir or Madam,

We would like to submit the attached manuscript for publication in Jove. For the following reasons, we believe that this paper will be of great interest to a wide range of researchers, including neuroscientists studying brain information processing, blood flow regulation, and disorders of cerebral blood flow.

(a) Cerebral vascular network, neurons and glial cells construct branches in three dimensions. It is therefore crucial to study their interaction in 3D with high spatial and temporal resolution. We implemented fast and repetitive volume scanning method in two-photon microscopy.

(b) Slow conducted vascular responses were recently discovered by the authors. The results were published in PNAS in 2018. It would be interesting to show how to repeat these experiments in details.

(c) In order to give localized and precise intervention to the blood vessels and nearby astrocytes, we optimized the procedure of glass micro-pipette insertion and local ejection (puffing).

(d) We used a new and advanced method to measure precisely vessel diameters: Chan-Vesse image segmentation and active contour algorithm. We showed how to conduct the measurement in details.

Yours sincerely,

Changsi Cai

10.23.2018

TITLE:

In Vivo Three-Dimensional Two-Photon Microscopy to Study Conducted Vascular Responses by Local ATP Ejection Using a Glass Micro-Pipette

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KEYWORDS:

in vivo, two-photon microscopy, cerebral blood flow (CBF), astrocytes, three dimensions (3D), neurovascular coupling, calcium imaging, viral vector injection

SUMMARY:

We present an optimized local ejection procedure using a glass micro-pipette and a fast two-photon hyperstack imaging method, which allows precise measurement of capillary diameter changes and investigation of its regulation in three dimensions.

ABSTRACT:

Maintenance of normal brain function requires a sufficient and efficient supply of oxygen and nutrition by a complex network of vessels. However, the regulation of cerebral blood flow (CBF) is incompletely understood, especially at the capillary level. Two-photon microscopy is a powerful tool widely used to study CBF and its regulation. Currently, this field is limited by the lack of in vivo two-photon microscopy studies examining (1) CBF responses in three-dimensions, (2) conducted vascular responses, and (3) localized interventions within the vascular network. Here, we describe a 3D in vivo method using two-photon microscopy to study conducted vascular responses elicited by local ejection of ATP with a glass micro-pipette. Our method uses fast and repetitive hyperstack two-photon imaging providing precise diameter measurements by maximal intensity projection of the obtained images. Furthermore, we show that this method can also be used to study 3D astrocytic calcium responses. We also discuss the advantages and limitations of glass micro-pipette insertion and two-photon hyperstack imaging.

INTRODUCTION:

The brain has a high energy consumption rate. About 20% of the oxygen and 25% of the glucose consumed by the human body are dedicated to brain function, while the brain only occupies 2% of the total body mass. Maintenance of normal brain function requires a sufficient and efficient supply of oxygen and nutrition by blood flow in a complex network of vessels. Local brain activity and cerebral blood flow (CBF) are robustly coupled, depending on the functional properties of neurons, astrocytes, pericytes, smooth muscle cells (SMCs) and endothelial cells (ECs)[1]. Recently, the first few orders of capillaries branching from penetrating arterioles have emerged as a 'hotspot'[2], showing active regulation of capillary blood flow. A slow conducted vascular response (CVR) was discovered at this 'hotspot' in mouse somatosensory cortex during both whisker stimulation and local ejection (puffing) of ATP with a glass micro-pipette[3].

Although in vivo imaging by two-photon laser scanning fluorescent microscopy has been widely used for studying neurovascular responses in cerebral cortex, most of the studies measured blood vessel diameters and investigated their regulation in a two-dimensional (2D) x-y plane. The challenges are: Firstly, cerebral blood vessels and their embracing astrocytes, pericytes and SMCs construct branches in three dimensions (3D). It is therefore crucial to study their interactions in 3D. Secondly, even a small amount of drift in focus will affect the precise measurement of both vessel diameters and cellular fluorescent signals. Finally, CVRs are fast and far-reaching in three dimensions. 3D volume scanning is optimal for detecting CVRs and unveiling their mechanisms. We implemented a piezo motor objective in a two-photon microscope to study mouse somatosensory cortex in vivo, allowing precise diameter measurements by maximal intensity projections of the obtained images.

Glass micro-pipettes have frequently been used for in vivo brain studies, e.g., to bulk-load organic dyes[4], record EEGs[5] and for patch clamping[6]. Nonetheless, limitations remain. Commonly, the tip of the glass micro-pipette is imprecisely placed, or the micro-pipette is not used for local interventions. Here, we have optimized the procedure of micro-pipette insertion and local ejection.

Furthermore, the combination of 3D two-photon microscopy and genetically-encoded fluorescent indicators offers an unprecedented opportunity to investigate neurovascular coupling in a 3D scope. In this study, we took advantage of this and injected viral vectors carrying astrocyte specific genetically-encoded calcium indicators into the mouse somatosensory cortex. Astrocytes as well as vessel diameters were imaged simultaneously by combining different fluorescent markers.

Overall, we present an optimized method of local ejection (puffing) by glass micro-pipette and fast two-photon hyperstack imaging, which allows precise measurement of capillary diameter changes. In addition, our method provides a novel tool to simultaneously study 3D profiles of Ca^{2+} responses in astrocytes and vascular diameter responses.

PROTOCOL:

All procedures involving animals were approved by the Danish National Ethics Committee according to the guidelines set forth in the European Council's Convention for the Protection of Vertebrate Animals Used for Experimental and Other Scientific Purposes and were in compliance with the ARRIVE guidelines.

1. Pre-surgical preparation

1.1. Clean the surgical table and all the surrounding area with 70% ethanol. Thoroughly clean and dry the surgical tools before the surgery.

1.2. Anesthetize an NG2DsRed mouse (Tg(Cspg4-DsRed.T1)1Akik/J; both genders; 4−8 months old) by a peritoneal injection of ketamine + xylazine (60 mg/kg + 10 mg/kg) dissolved in sterilized water, pH 7.4. Administer supplemental doses of ketamine (30 mg/kg) every 25 min until completion of the surgical procedure. Check tail and toe pinch reflexes regularly to verify the depth of anesthesia.

1.3. Inject saline subcutaneously at four different locations on the back of the mouse for a total volume of 1 mL to prevent dehydration during the experiment. To protect the eyes from drying out, apply eye lubricant. Maintain body temperature at 37 °C using a rectal temperature probe and heating blanket.

2. Surgical procedure

2.1. Tracheotomy

2.1.1. Place the mouse on its back. Inject 0.04 mL of 0.5% lidocaine subcutaneously under the planned incision and wait for 2 min. Make a 10 mm long incision above the chest bone (manubrium). Separate the submandibular glands and sternothyroid muscles, and then expose the trachea.

2.1.2. Make tracheostomy between two tracheal rings with scissors. Insert a small metal tube with a length of 16.2 mm and a diameter of 1 mm into the trachea. Connect the tube to a mechanical ventilator and capnograph to monitor the end-expiratory CO_2 (**Figure 1A**).

2.2. Catheter insertion

2.2.1. Inject 0.5% lidocaine (0.04 mL) subcutaneously under the planned incision and wait for 2 min. Make an incision in the inguinal area of the left leg. Bluntly dissect the connective tissue until the femoral artery and vein are exposed.

2.2.2. Using fine tip forceps gently separate the artery from the vein. Stop the blood flow upstream with a microvascular clamp. Constrict the blood flow downstream with a 10-0 nylon suture ligating both blood vessels.

2.2.3. Use micro-dissecting scissors to make a small incision in both the artery and the vein. Insert plastic catheters into both blood vessels. Secure the catheters by tightening the sutures (8-0 or 10-0 nylon sutures dependent on the vessel size) without compromising catheter lumens. Remove the microvascular clamp and close the skin.

2.2.4. Monitor blood pressure via the arterial catheter. Measure levels of blood gases in arterial blood samples (50 µL) before and after each experiment (normal values: pO_2, 90–120 mmHg; pCO_2, 35–40 mmHg; pH, 7.25–7.45) using a blood gas analyzer. Use the vein catheter for infusion of fluorescein and anesthesia.

2.3. Craniotomy

2.3.1. Shave the fur off the head of the mouse between the ears. Inject 0.04 mL of 0.5% lidocaine subcutaneously under the planned incision and wait for 2 min. Remove the scalp, completely exposing the parietal bones and bregma on both sides.

2.3.2. Wipe the skull using a 10% iron-chloride solution to remove the periosteum. Rinse the skull thoroughly with saline. Glue a metal head bar to the skull with cyanoacrylate glue and activator (**Figure 1A**).

NOTE: The metal head bar is 75 mm long and 13.5 mm wide, with a round hole of 5 mm diameter in the center.

2.3.3. Drill a 4-mm-diameter craniotomy, centered at 0.5 mm behind and 3 mm to the right of the bregma over the right sensory barrel cortex. Remove the dura with a fine-tip vessel dilator.

2.3.4. Cover the exposed cortex with 0.75% agarose gel, dissolved in artificial cerebrospinal fluid (aCSF; pH = 7.4) and cooled to 35 °C. Cover 80–90% of the craniotomy with a glass coverslip at an angle of 10–15° to the metal head bar (**Figure 1B**) that permits insertion and placement of the glass micro-pipette.

2.3.5. To minimize brain pulsation, glue the two corners of the glass coverslip onto the metal head bar with cyanoacrylate glue and activator. Apply the activator carefully to prevent getting onto the exposed brain. Rinse the glued glass coverslip thoroughly with saline afterwards.

2.4. Perform insertion of the whisker pad stimulation probe. Insert a set of custom-made bipolar electrodes (8 mm length and 0.25 mm thickness) percutaneously into the left side of face to stimulate the ramus infraorbitalis of the trigeminal nerve contralateral to the craniotomy. Position the cathode close to the hiatus infraorbitalis, and insert the anode into the masticatory muscles[7]. Perform the stimulation with an electrical stimulator at an intensity of 1.5 mA for 1 ms in trains of 20 s at 2 Hz.

2.5. Upon completion of surgery, administer a bolus of 0.05 mL fluorescein isothiocyanate-dextran (FITC-dextran, 4% w/v, molecular weight [MW] 50,000) into the femoral vein to label the

blood plasma. Discontinue ketamine and switch the anesthesia to continuous intravenous infusion of α-chloralose (17% w/v, final concentration) mixed with FITC-dextran (2% w/v, final concentration) at 0.02 mL/10 g/h. Wait for about half an hour for stabilization of the new anesthetic state.

NOTE: Both FITC-dextran and α-chloralose are dissolved in 0.9% saline.

3. First two-photon imaging session

3.1. Transfer the mouse to the stage of a commercial two-photon microscope (**Table of Materials**). Under both red fluorescent protein (RFP, **Figure 1C**) and green fluorescent protein (GFP, **Figure 1C**) modes of epi-fluorescent illumination, take a picture of the exposed cortex with a 5x objective. Use the RFP picture as a 'map' for insertion of the glass micro-pipette in the next session.

NOTE: The GFP 'map' is used to distinguish veins/venules from arteries/arterioles.

3.2. Perform two-photon imaging using the two-photon microscope and a 25x 1.0 numerical aperture (NA) water-immersion objective with piezo motor. Set the excitation wavelength to 900 nm. Search the cortex and follow each penetrating arteriole and find its horizontal branches (1st order capillaries).

3.3. Image penetrating arterioles and their 1st order capillaries at rest and during whisker pad stimulation. See the detailed imaging parameters in section 5.

3.4. Mark locations of 1st order capillaries with >5% vasodilation during whisker pad stimulation on the RFP 'map' (**Figure 1C**). Locations with <5% vasodilation are considered as being outside of the whisker barrel cortex region.

NOTE: If using wild-type mice instead of NG2DsRed mice in the experiment, the GFP image is used as 'map' instead.

4. Insertion of the glass micro-pipette

4.1. Prepare glass micro-pipettes for puffing using a pipette puller (**Table of Materials**). The glass micro-pipettes have a resistance of 3–3.5 MΩ and a taper length of 4.5–5 mm. Load a pipette with a mixture of 1 mM ATP and 10 µM Alexa 594 in order to visualize the pipette tip under the epi-fluorescent lamp and two-photon microscope.

4.2. Mount the glass micro-pipette onto a patch clamp holder and connect it to an air pump. Set the pump holding pressure to 0 psi.

4.3. Using red epi-fluorescent illumination, focus on the pipette tip with the 5x objective. Place the glass micro-pipette into the aCSF above the agarose. Adjust the holding pressure of the air

pump to 0.2 psi. A small red cloud can be observed ejecting out of pipette tip in the aCSF. This is to prevent clogging during pipette insertion.

4.4. Choose one of the marked locations in the RFP 'map' as destination. Move the pipette tip to the horizontal plane of the cover glass edge with 30 µm distance, roughly pointing towards the destination in the x-y plane.

4.5. Carefully lower the pipette tip in z-axis under the cover glass slip. Then start advancing the glass pipette towards the destination until ~500 µm above the brain surface. Switch focus frequently between the cover glass edge, the pipette tip and the brain surface, making sure there is enough free space for moving the pipette.

4.6. Switch the objective to 25x. Re-center the pipette tip and advance the pipette tip further towards the destination on the RFP 'map'. Keep the pipette tip in the agarose layer.

4.7. When the pipette tip is ~100 µm above the brain surface, switch the imaging mode to two-photon microscopy. Focus on a target location at which to puff. Write down its x, y coordinates (x_0, y_0) and its depth below the surface (z_0). Calculate the coordinates of the insertion point on the brain surface (x_i, y_i) as follows:

$$x_i = x_0 + z_0/\tan\theta$$
$$y_i = y_0$$

where **θ** is the angle between the pipette holder and the horizontal plane.

4.8. Position the pipette tip to the coordinates (x_i, y_i) on the brain surface. Gently and slowly insert the pipette until it reaches (x_0, y_0, z_0). If a vessel is in the way of an insertion path, withdraw the pipette and re-calculate other insertion coordinates and path; or turn the stage plate slightly (as indicated in **Figure 1A**).

4.9. Set the pump holding pressure at 0 psi and ejection pressure at 10–15 psi. Puff ATP for 200–400 ms at the target location during two-photon imaging. Adjust the pressure and duration of puffing for each pipette and over time as explained in the discussion section.

5. Hyperstack two-photon imaging

5.1. Perform experiments using the two-photon microscope and a 25 x 1.0 NA water-immersion objective with piezo motor. Set the excitation wavelength to 900 nm. Filter the emitted light to collect red light from DsRed (pericytes)/tetramethylrhodamine isothiocyanate-dextran (TRITC-dextran) staining blood plasma and green light from FITC-dextran (blood plasma)/GCaMP6 (GFP, astrocytic calcium).

5.2. Produce a high-resolution z-stack at the location of interest containing a penetrating arteriole, a 1st order capillary, a 2nd order capillary and possibly neighboring fluorescent cells (e.g., pericytes, astrocytes). Determine the x*y*z volume of hyperstack imaging by including the 3D

structure of the blood vessels as much as possible. The total height of each stack may vary from 30−50 µm, while the x-y plane roughly covers an area of 60 µm x 40 µm.

5.3. Set the image stack in the imaging software to be comprised of 8−10 planes with an inter-plane distance of 4−5 µm. The piezo-motor objective stops at each level on the z-axis to acquire the image of each plane. The sampling rate is 1 s per stack and pixel resolution in the x-y plane is 0.2−0.3 µm (**Figure 2A**). One recording normally includes 10 pre-puff image stacks and 150 post-puff image stacks.

6. Data processing

6.1. Process data using custom-made analytical software. Flatten each image stack into one image by maximal intensity projection (**Figure 2B**). Draw a rectangular region of interest (ROI) with a width of 3 µm perpendicularly across the vessel of interest (**Figure 2C**).

6.2. To minimize interference from shadows of red blood cells and from minor vibrations of the cortex, average the rectangular ROI by projecting it into one line, which then represents the average profile of the vessel segment. Do this for each maximal intensity image.

6.3. Plot the profile lines as a 2D image with the x-axis representing maximal intensity images in chronological order (**Figure 2D**, upper panel). Use an active contour algorithm (Chan-Vese segmentation) to find the edges of the vessel[8,9] (indicated by red curves; **Figure 2D**, upper panel).

6.4. Calculate the time course of the diameter changes as the difference between the edges of the blood vessel (vertical distance between the upper and lower red curves in **Figure 2D**, lower panel). Normalize diameter changes to pre-puffing diameter baseline and plot curves of diameter change over time. Do this for each ROI (**Figure 2E**).

6.5. The vessel response amplitude is defined as the highest/lowest peak amplitude after puffing. The response latency is defined as the latency of half-max amplitude (**Figure 2G**). Manually trace the skeletonized vascular structure by placing nodes along the vessels (**Figure 2F**) and measuring geographical distance between each ROI and the penetrating arteriole. Calculate CVR speed by dividing the distances by latency differences.

7. Viral vector injection

7.1. In order to simultaneously study astrocytic calcium responses, inject a volume of 0.6 µL viral vector (pZac2.1 gfaABC1D-lck-GCaMP6f, Addgene) into the somatosensory cortex of NG2DsRed mice, following the standard stereotactic viral vector injection procedure[10].

7.2. After three weeks, astrocytes in mouse somatosensory cortex express the genetically encoded calcium indicator, GCaMP6f. Follow the aforementioned surgical procedure and two-photon imaging for mice. To distinguish between astrocytes and vessel lumina, TRITC-dextran instead of FITC-dextran is used to stain vessel lumina red.

REPRESENTATIVE RESULTS:

Once the surgery was complete, mice were transported to two-photon microscope (**Figure 1A**). A glass micro-pipette containing 1 mM ATP was inserted in proximity of the destination blood vessel at the target location (**Figure 1B**).

We performed hyperstack imaging while giving a puff of 1 mM ATP (**Figure 2A**, **Supplementary Video 1**). Each image stack was flattened to one image by maximal intensity projection (**Figure 2B**). Rectangular ROIs were placed perpendicularly across the vessel to measure vessel diameter change upon ATP puffing (**Figure 2C**). Vessel diameters were measured using Chan-Vese segmentation (**Figure 2D**). In single puff recordings, normalized diameter changes at each ROI were overlaid to compare the responses of different vessel segments (**Figure 2E**). The distance from each ROI to the penetrating arteriole was calculated by hand-drawing the vascular skeleton (**Figure 2F**). Amplitudes and latencies of dilation and constriction at each ROI in single puff recordings were plotted over the calculated distances from the ROIs to the penetrating arteriole (**Figure 2H-K**). Vasodilation upon puffing propagated linearly with a speed of 14.69 µm/s (upstream) and of 2.8 µm/s (downstream), starting from the junction of 1st and 2nd order capillaries (**Figure 2H**). Vasoconstriction also propagated linearly at 3.92 µm/s, starting from the penetrating arteriole (**Figure 2I**). Maximal amplitude of both vasodilation and vasoconstriction occurred in 1st order capillaries (**Figure 2J-K**).

Furthermore, combining astrocyte-specific viral vector-carrying fluorescent calcium indicators and two-photon hyperstack imaging, astrocytic calcium responses to ATP puffing were investigated (**Figure 3A**, **Supplementary Video 2**). Rectangular ROIs were placed at astrocytic somata and processes. ATP induced a rise in intracellular calcium in astrocytic processes but not in astrocyte somata (**Figure 3B**).

FIGURE LEGENDS:

Figure 1: Diagram of in vivo two-photon 3D imaging and glass micropipette puffing. (**A**) The anesthetized mouse is head-fixed to a metal bar and mechanically ventilated. End-tidal CO_2 is monitored by a capnograph. Both whisker pad stimulation and micro-pipette puffing are used to induce vascular diameter changes. The glass micro-pipette holder is mounted on the stage plate. The femoral artery and vein are catheterized to monitor blood pressure and blood gases, and to infuse anesthesia and fluorescein, respectively. (**B**) The glass coverslip covers the craniotomy at an angle, which allows free movement of the pipette. The puffing micro-pipette is placed in proximity of a penetrating arteriole and its capillaries and contains a mixture of 10 µM Alexa 594 (red color in glass micro-pipette) and 1 mM ATP. The two-photon imaging is a fast and repetitive 3D volume scanning that includes the penetrating arteriole and the first few order capillaries, as well as neighboring astrocytes and pericytes. (**C**) Representative images of red fluorescent protein (RFP) and green fluorescent protein (GFP) using epi-fluorescent illumination are shown. They are used as 'maps' during pipette insertion. Red circles mark locations of 1st order capillaries with >5% vasodilation during whisker pad stimulation. Scale bar: 50 µm.

Figure 2: ATP puffing by micro-pipette induces vessel dilation, followed by constriction. (**A**) Cascade of planes in one representative image stack including penetrating arteriole and 1st and 2nd order capillaries. Pericytes are labeled with a red fluorophore (NG2-DsRed) and the vessel lumen is labeled with FITC-dextran (green). (**B**) Maximal intensity projection of the image stack. (**C**) Multiple uniquely colored regions of interest (ROIs) placed perpendicularly across the vasculature to measure the vessel diameter. (**D**) Top, representative fluorescent intensity over time at the dark blue ROI from panel C. The two red curves define the edges of the vessel wall. The vertical distance between the two red curves is the vessel diameter and is shown as a function of time (bottom). (**E**) Normalized diameter changes over time at each ROI in response to 1 mM ATP puffing. Measurements are based on a single experiment. (**F**) The vascular skeleton was manually traced by placing nodes along the vessels. (**G**) Amplitudes of dilation or constriction were defined as maximal positive or negative vascular response, respectively, during the recording session. The latency of dilations and constrictions were reported as time to half positive or negative maximum after puffing onset. (**H-K**) Graphs show the latency of dilation (**H**), the latency of constriction (**I**), the amplitude of dilation (**J**), and the amplitude of constriction (**K**) of all the ROIs shown in panel C versus the distance of each ROI from the penetrating arteriole. The dashed lines represent the linear fitting of upstream and downstream conductive responses. p.a.: penetrating arteriole. Scale bar: 10 μm.

Figure 3: ATP puffing by micro-pipette induces astrocytic calcium activities. (**A**) Astrocytic viral vectors carrying a fluorescent calcium indicator were injected in mouse somatosensory cortex three weeks prior to the experimental procedure. Image shows vessel lumina labeled with TRITC-dextran (red) and astrocytic calcium in green. (**B**) Multiple ROIs are placed at astrocytic somata and processes. Upon 1 mM ATP puffing, intracellular calcium increased in astrocytic processes but not in somata (dotted boxes), where calcium levels larger than the mean plus 1.5 x standard deviation were defined as significant. Scale bar: 10 μm.

Supplementary Video 1: Time-series movie flattened from hyperstack imaging of vessels in response to puffing of 1 mM ATP. Green: FITC-dextran, staining vessel lumen. Red: NG2DsRed, staining pericytes.

Supplementary Video 2: Time-series movie flattened from hyperstack imaging of astrocytic calcium in response to puffing of 1 mM ATP. Green: astrocytic calcium. Red: TRITC-dextran, staining vessel lumen.

DISCUSSION:

One challenge for vascular studies is the precise measurement of vessel diameters. The method we describe here used a motorized piezo objective to make fast and repetitive hyperstack imaging by two-photon microscopy. Firstly, this method allows repeated examinations of the penetrating arteriole, 1st order and 2nd order capillaries without loss of focus and led to the discovery of slowly conducted vascular responses in capillaries in vivo. Secondly, in combination with a viral vector injection technique, it enables us to investigate astrocytic calcium responses in three dimensions, which is necessary for studies of blood flow regulation.

This method is not without limitations. Firstly, a narrow rectangular field of view is defined before the 3D imaging in order to achieve high temporal resolution. This usually limits the imaging to first three orders of capillaries. Secondly, lasers of two-photon microscopes must be perfectly aligned. Laser misalignment will falsely de-center each frame and increase the vessel diameter measurement. Thirdly, the sampling rate of 3D imaging is capable of capturing slow CVRs and slow calcium changes in astrocytes. 1-2 Hz per stack is still not fast enough to study fast CVRs[11] or fast calcium signals in astrocytes[12,13].

Furthermore, in order to affect vessels of interest locally, we have optimized the procedure of precise insertion of a glass micro-pipette into a specific location of the mouse cerebral cortex. There are several critical steps for successful application of this method. Firstly, the angle of the glass micro-pipette holder must be carefully adjusted. It should allow the micro-pipette to freely maneuver beneath the objective and the glass coverslip above the exposed cortex. Secondly, during insertion of the glass micro-pipette into the agarose layer and the cortex, a small holding pressure is necessary to keep the pipette tip unclogged. However, caution must be exerted not to apply too great a holding pressure so that the compound does not leak before puffing. Thirdly, the puffing of the pipette should be tested in agarose layer above the brain to find the optimal pressure and duration releasing enough puffing compound upon one puff while not introducing an obvious displacement of cells and vessels due to mechanical pressure.

In order to make more precise measurement of CVR speed, volume scanning speed should be improved. There are other newly-developed volume-scanning microscopes, for example, a two-photon microscope with an acoustic optical deflector (AOD) scans as fast as 5 volumes per second with the same spatial resolution and volume size (email communication). Light-sheet microscopes scan even a larger volume size (350 µm x 800 µm x 100 µm) with 10 volumes per second[14].

Our mouse preparation also has limitations. Acute mouse surgery may have potential complications. Pain relief medicine and anesthesia may affect the neurovascular responses. Acute neuroinflammation can also be triggered, resulting in microglial activation and leukocytosis in cortical tissues and vessels. Although the tip size of glass micro-pipettes is usually very small (<1 µm), the insertion procedure and ATP puffing could also potentially trigger microglial migration and embracement of insertion site within 0.5−1 hour post-insertion.

In conclusion, combination of 3D imaging in two-photon microscopy and localized puffing glass micro-pipette is an advanced tool to study neurovascular activities and their mechanism.

ACKNOWLEDGEMENTS:

This study was supported by the Lundbeck Foundation, the NOVO-Nordisk Foundation, the Danish Council for Independent Research | Medical Sciences, and the NORDEA Foundation Grant to the Center for Healthy Aging.

DISCLOSURES:

The authors have nothing to disclose.

REFERENCES:

1. Abbott, N. J., Patabendige, A. A., Dolman, D. E., Yusof, S. R., Begley, D. J. Structure and Function of the Blood-Brain Barrier. *Neurobiology of Disease*. **37** (1), 13−25 (2010).
2. Hall, C. N. et al. Capillary Pericytes Regulate Cerebral Blood Flow in Health and Disease. *Nature*. **508** (7494), 55−60 (2014).
3. Cai, C. et al. Stimulation-Induced Increases in Cerebral Blood Flow and Local Capillary Vasoconstriction Depend on Conducted Vascular Responses. *Proceedings of the National Academy of Sciences of the United States of America*. **115** (25), E5796−E5804 (2018).
4. Stosiek, C., Garaschuk, O., Holthoff, K., Konnerth, A. In Vivo Two-Photon Calcium Imaging of Neuronal Networks. *Proceedings of the National Academy of Sciences of the United States of America*. **100** (12), 7319−7324 (2003).
5. Mathiesen, C. et al. Activity-Dependent Increases in Local Oxygen Consumption Correlate with Postsynaptic Currents in the Mouse Cerebellum in Vivo. *The Journal of Neuroscience*. **31** (50), 18327−18337 (2011).
6. Kitamura, K., Judkewitz, B., Kano, M., Denk, W., Hausser, M. Targeted Patch-Clamp Recordings and Single-Cell Electroporation of Unlabeled Neurons in Vivo. *Nature Methods*. **5** (1), 61−67 (2008).
7. Norup Nielsen, A., Lauritzen, M. Coupling and Uncoupling of Activity-Dependent Increases of Neuronal Activity and Blood Flow in Rat Somatosensory Cortex. *The Journal of Physiology*. **533** (3), 773−785 (2001).
8. Wang, X. F., Huang, D. S., Xu, H. An Efficient Local Chan-Vese Model for Image Segmentation. *Pattern Recognition*. **43** (3), 603−618 (2010).
9. Chan, T. E., Sandberg, B. Y., Vese, L. A. Active Contours without Edges for Vector-Valued Images. *Journal of Visual Communication and Image Representation*. **11** (2), 130−141 (2000).
10. Cetin, A., Komai, S., Eliava, M., Seeburg, P. H., Osten, P. Stereotaxic Gene Delivery in the Rodent Brain. *Nature Protocols*. **1** (6), 3166−3173 (2006).
11. Chen, B. R., Kozberg, M. G., Bouchard, M. B., Shaik, M. A., Hillman, E. M. A Critical Role for the Vascular Endothelium in Functional Neurovascular Coupling in the Brain. *Journal of the American Heart Association*. **3** (3), e000787 (2014).
12. Lind, B. L., Brazhe, A. R., Jessen, S. B., Tan, F. C., Lauritzen, M. J. Rapid Stimulus-Evoked Astrocyte Ca2+ Elevations and Hemodynamic Responses in Mouse Somatosensory Cortex in Vivo. *Proceedings of the National Academy of Sciences of the United States of America*. **110** (48), E4678−4687 (2013).
13. Stobart, J. L., Ferrari, K. D., Barrett, M. J. P., Gluck, C., Stobart, M. J., Zuend, M., et al. Cortical Circuit Activity Evokes Rapid Astrocyte Calcium Signals on a Similar Timescale to Neurons. *Neuron*. **98** (4), 726 (2018).
14. Bouchard, M. B., Voleti, V., Mendes, C. S., Lacefield, C., Grueber, W. B., Mann, R. S., et al. Swept Confocally-Aligned Planar Excitation (Scape) Microscopy for High Speed Volumetric Imaging of Behaving Organisms. *Nature Photonics*. **9** (2), 113−119 (2015).

Figure 1

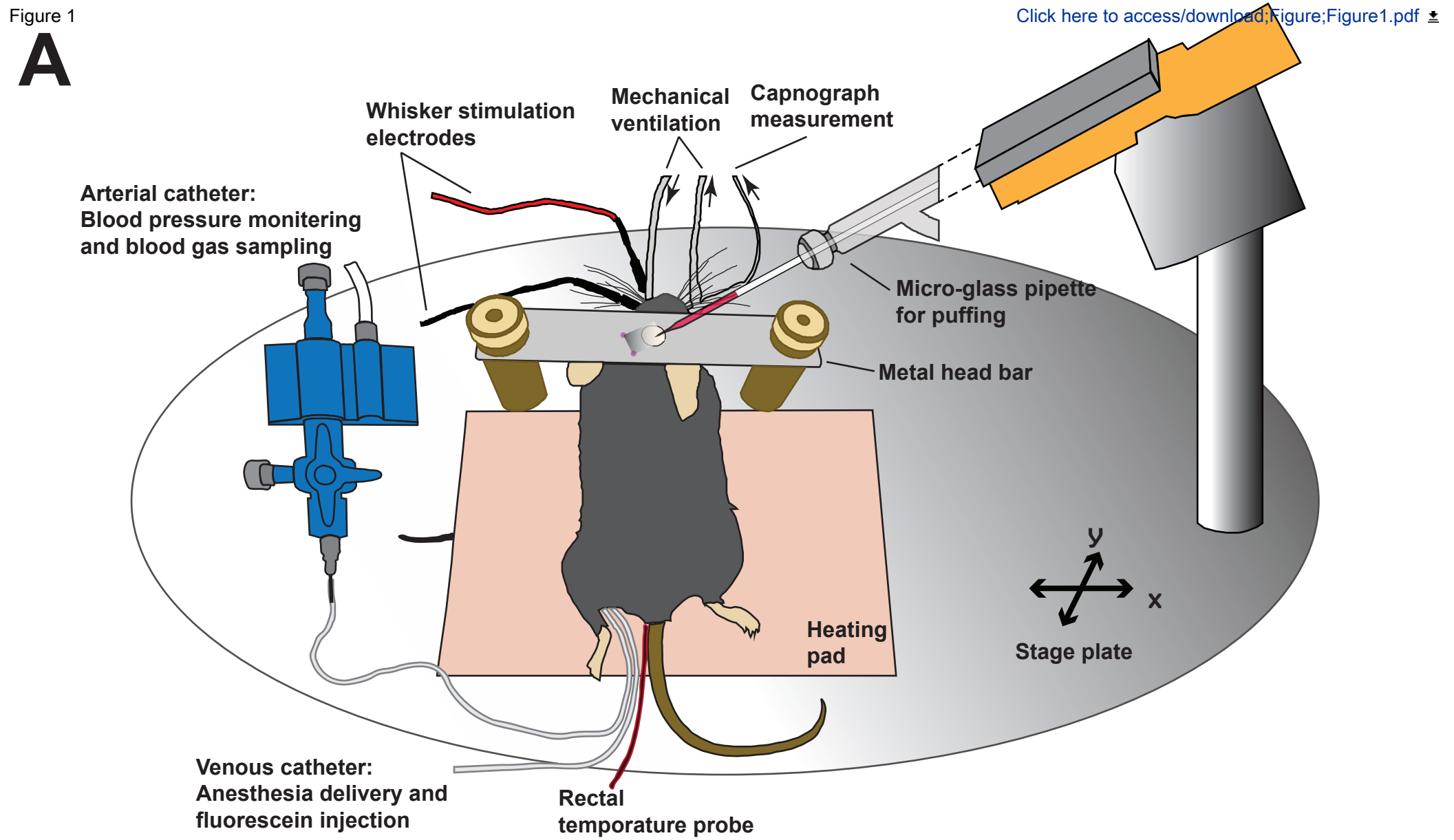

Figure 2

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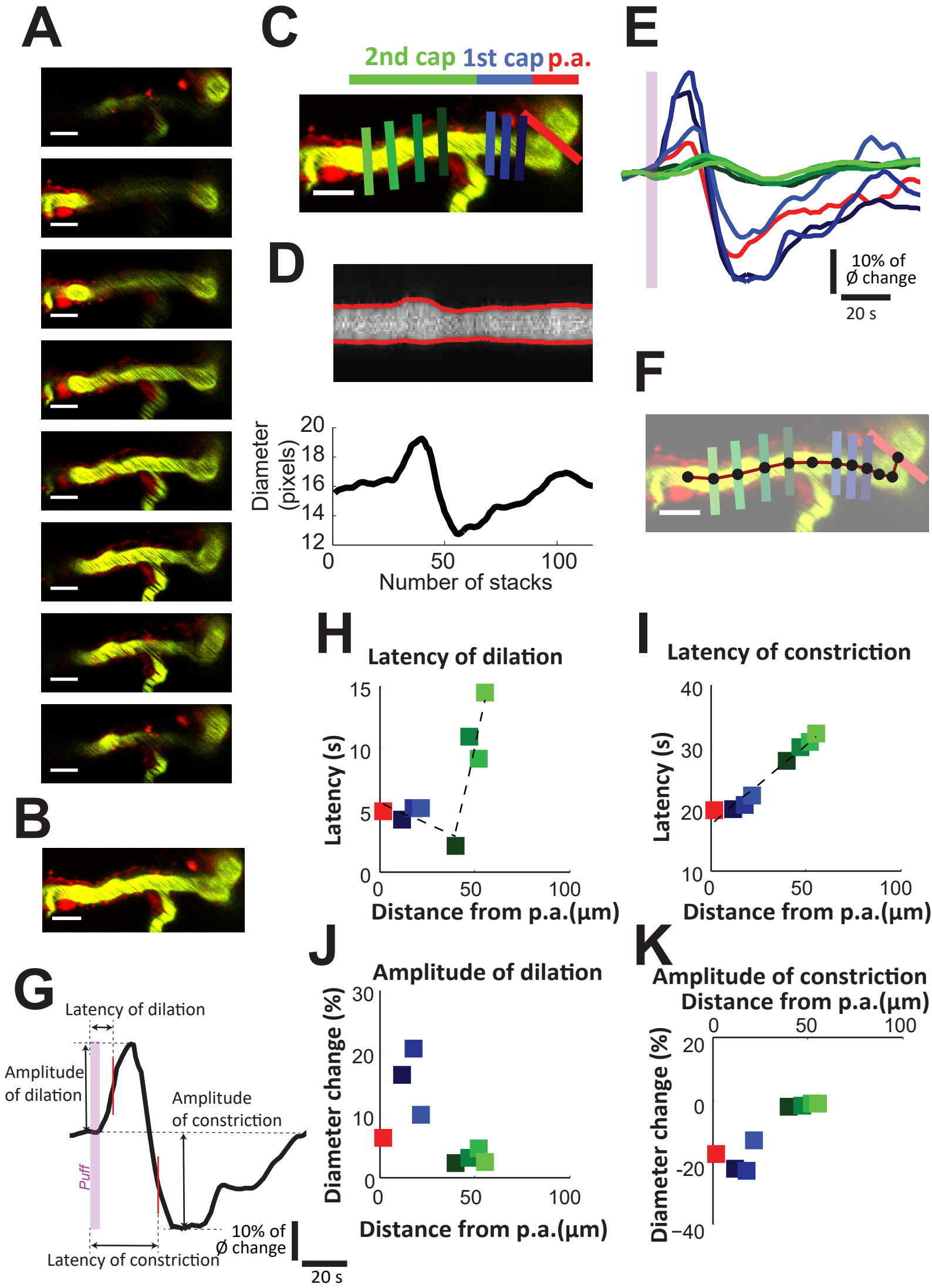
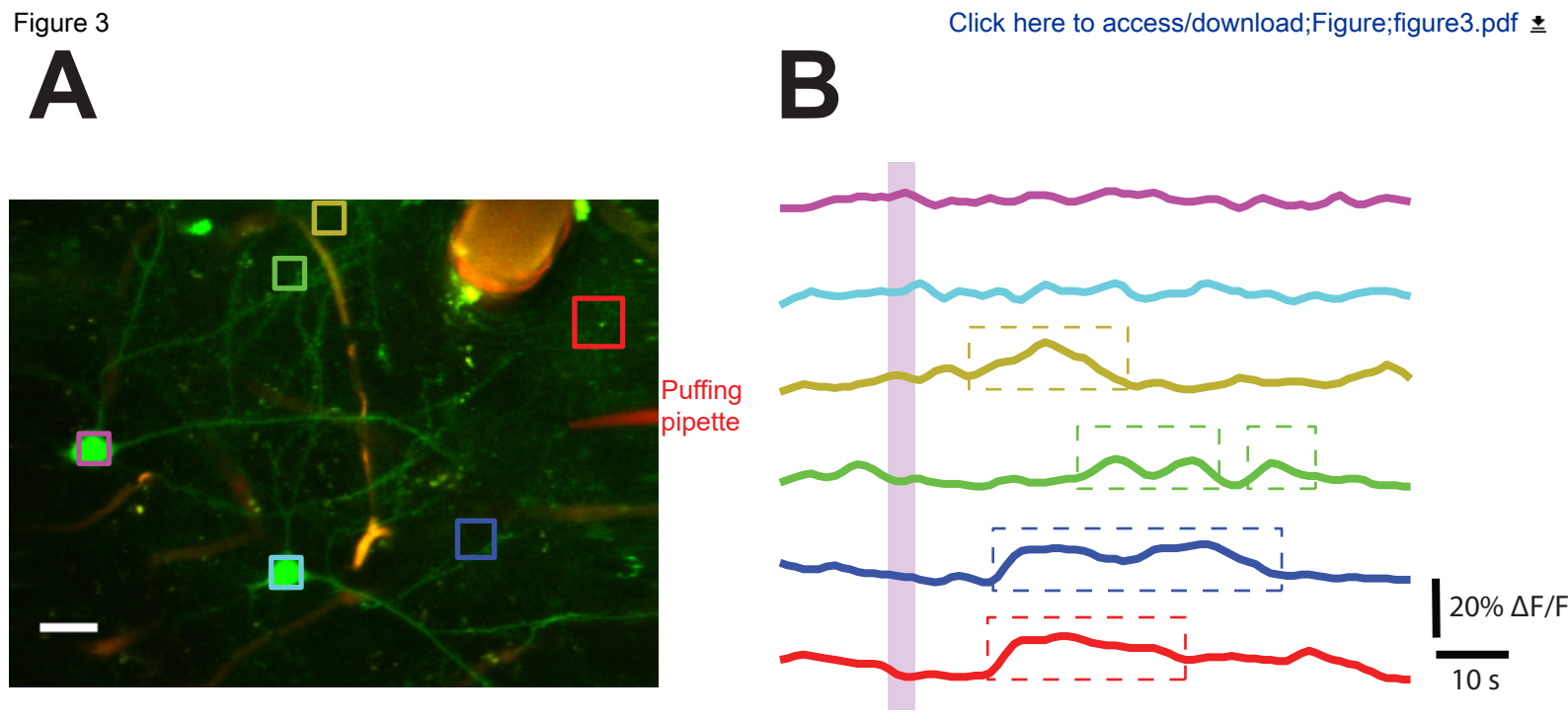
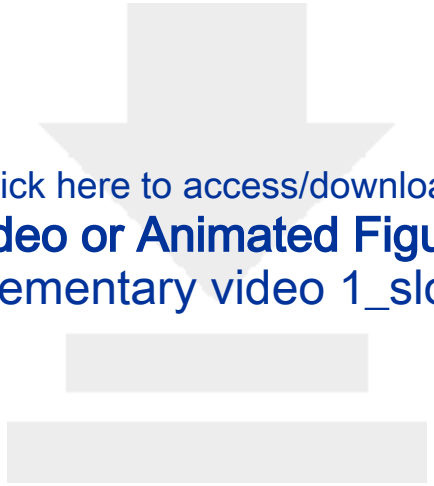


Figure 3

Click here to access/download;Figure;figure3.pdf



Supplementary Video 1



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Video or Animated Figure

Supplementary video 1_slow.avi

Supplementary Video 2

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Supplementary video 2_slow.avi

List of Materials	Company	Catalog Number
Agarose	Sigma–Aldrich	A6138
Alexa 594	Life Technologies	A-10438
ATP	Sigma-Aldrich	A9187
Cyanoacrylate glue and activator	Loctite	Adhesives and SF7452
Eye lubricant	Neutral Ophtha, Ophtha A/S, Denmark	
FITC-dextran	Sigma-Aldrich	FD500S
NG2DsRed mice	Jackson Laboratory	8241
pZac2.1 gfaABC1D-lck-GCaMP6f	Addgene	52924-AAV5
TRITC-dextran	Sigma-Aldrich	52194

List of Equipments	Company	Catalog Number
Air pump	WPI	PV830
Blood gas analyzer	Radiometer	ABL 700
Blood pressure monitor	World Precision Instruments	BP-1
Body temperature controller	CWE	Model TC-1000
Capnograph	Harvard Apparatus	Type 340
Electrical stimulator	A.M.P.I.	ISO-flex
Mechanical ventilator	Harvard Apparatus	D-79232
Micropipette puller	Sutter Instrument	P-97
Two-photon microscope	Femtonics Ltd	Femto3D-RC

List of Surgical Instruments	Company	Catalog Number
Anatomical tweezer	Lawton	09-0007
Angled and balanced tweezer	S&T AG	00595 FRAS-18 RM-8
Iris scissor	Lawton	05-1450
Micro vascular clamp	S&T AG	462
Mouse vascular catheters	Verutech	100828
Straight and balanced tweezer	S&T AG	00361 FRS-18 RM-8.1

Tubing introducing forcep	S&T AG	TIF04
Vannas scissor	S&T AG	SAS-15 R8T
Vessel dilator	S&T AG	D-5a.1

Comments/Description
Apply upon exposed cortex for protection
Stain puffing compound to red fluorescent color
Vasodilator and vasoconstrictor, puffing compound
Glue for metal piece and coverglass
Keep the mouse eyes moisterized
Blood serum dye, green fluorescent color
These transgenic mice express an red fluorescent protein variant (DsRed) under the control of the mouse NG2 (Cspg4) promoter
Astrocyte specific viral vectors carrying genetically encoded calcium indicators
Blood serum dye, red fluorescent color

Comments/Description
Give air pressure to pipette puffing
Measure levels of blood gases
Monitor aterial blood pressure
Keep the mouse body temperature in physiological range
Monitor the end-expiratory CO_2 from the mouse
Apply whisker pad stimulation
Mechanically ventilate the mouse in physiological range

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Title of Article: A 3D in vivo method to study conducted vascular responses by glass micro-pipette puffing in mouse cerebral cortex by two-photon microscope

Author(s): Chongxi Cai, Stefan Zambach, Jonas Fordsmann, Micael Lonstrup, Aske Jenson, Martin Lauritzen

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Article Title: A 3D in vivo method to study conducted vascular responses by glass micro-pipette puffing in mouse cerebral cortex by two-photon microscope

Signature: Changsi Cai Date: 23/10/2018

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We thank all reviewers for their comments, which have helped greatly in the preparation of an improved manuscript.

Please see below a point-by-point response to each of the comments. The reviewers' comments are marked with shaded background. The changes are marked as red color in the text. The replies to reviewers are colored as blue. We have expanded Figure1, and the language is improved by a native English speaker.

We have also added one co-author, Kirsten Joan Thomsen, who has helped us improve the language and gave us many technical suggestions.

Reviewer #1:

Manuscript Summary:
The authors provide a methods video/paper for their recent publication (PNAS 2018) on conducted vascular responses. Combining two-photon imaging of small structures (capillaries, astrocytic processes) with pipette puffing of compounds in living animals is a challenging technique and so this publication will be a benefit to the field. The authors describe all facets of their experiment - including the initial surgery, the craniotomy, how to place the coverglass so that a pipette can be inserted under it, the difficult task of guiding the pipette to the targeted region, and how they merge the stack to compute vascular or astrocytic dynamics over a small volume. In my opinion, all the details necessary for others to duplicate the procedure have been provided and the paper is well organized. I have only a few minor questions.

Major Concerns:
none

Minor Concerns:

Because the image stack is taken over around 1 seconds, segments from top frames will have been collected earlier in time from segments in lower frames. Perhaps the volume is small enough so that many frames go into the instantaneous diameter calculation. But I would like to hear if the authors have any comment about the possibility that this could affect the latency computations. For instance if a vessel in one of the upper frames of the stack began to dilate just after its frame was collected, it would not be imaged again for close to one second and a vessel deeper in the stack might look like it had dilated sooner but that would only be because of the time when it was sampled.

Thanks for pointing this out. We agree that it will be more precise to use the time of each plane in the stack to calculate the real latency of vaso-responses. And we are right now implementing this function in our customized analyzing software.

In the meanwhile, we still believe that the slow conducted vascular responses exist. In our PNAS paper, we also observed the same phenomena by simple 2D time-series scanning. And our hyperstack imaging avoids the possibilities of focus drift which leads to better estimation of vessel diameters. Secondly, each vasculature has a unique structure. If there is a direct correlation of response latency and z-position in the image stack for each ROI, we would expect to see the earliest responding ROI located at the first plane of each stack. But actually

it is not the case in our study.

The authors mention checking puffing parameters for pipettes in other regions of cortex. Does this need to be done for every pipette or do you get a general feel for what are good parameters after some practice and then use these during experiments? If it does need to be done every time, does positioning it in one region of cortex and then withdrawing and moving to the target region cause any problems - instability by moving around in the agar, or potentially clogging the tip with a second insertion? Perhaps the authors could provide more detail about how this step was mastered.

In the beginning phase of establishing this method, we indeed tested the puffing parameters in the other region of cortex before the formal experiment. But now, we normally give a gentle puff inside agarose before insertion, and check whether a red cloud is instantaneously observed around the pipette tip with 25x objective. This puffing location should be far away from the regions of interest for puffing experiments (0.5-1 mm above surface). The newly produced glass micro-pipettes have consistent resistance of 3-3.5mΩ. This usually conveys smooth puffing experiments. But the tip may get clogged if storing for a long time. This is the reason of testing inside agarose.

We have therefore removed the word 'test in the other region of cortex' in the discussion part.

This is a methods paper and so the scientific significance of results is probably not at stake. But I do wonder what the physiological significance of such a slowly propagating signal could be. Similarly, the astrocyte processes can take more than 10 seconds to respond to a puff of ATP right next to them. Were other compounds (which can generate much more rapid vascular responses) not chosen because the method isn't fast enough to resolve rapid conduction or was there a special reason for choosing ATP?

Thanks for this brain-storming question. We think the slow CVR could possibly facilitate functional vasodilation instead of playing active roles in dilation onset. Furthermore, please note that the definition of latency is time to half of maximal vaso-responses, and isn't the real onset time. During the data analysis, we realized that linear propagation pattern is the most obvious when calculating half-peak latency. This is due to the temporal properties of our two-photon hyperstack imaging.

ATP is an important signaling molecule in purinergic signaling, which could is involved in astrocyte-astrocyte, astrocyte-pericyte crosstalk, regulating of blood flow in arterioles and capillaries. This is our motivation to use ATP and its analogs. There are also other reasons to choose ATP: (1) ATP induces vasodilation followed by vasoconstriction; while most of other vasoactive compounds could only induce monophasic effect. (2) ATP is very easy to degenerate and basic vascular tune returned to baseline quickly (within 10 minutes).

Reviewer #2:

Manuscript Summary:
The article describes a protocol to acquire in vivo rapid two photon images stacks of the brain vasculature in mice and more importantly the ability to simultaneously deliver a puff of ATP through a precisely localized micropipette. In addition Ca2+ imaging of astrocytes response is obtained possible after ad hoc viral injection. The technique requires a two photon microscope with a objective mounted on a piezo motor.

General remarks:

In the introduction some precisison should be added to quantifiy "high spatial and temporal resolutions" and the dimensions of the 3D volume that is actually imaged.

Thanks for the comment. Regarding the spatial resolution, we currently use pixel resolution of 0.2-0.3 µm, which is sufficient to study the calcium signaling in somata and processes of astrocytes, as well as vessel diameters. Regarding the temporal resolution, we currently use 1s per stack. This is sufficient to study the slow calcium activities in astrocytic compartments and slow CVRs. However, the recent findings about the fast calcium activities in astrocytes are in a similar temporal scale with neurons, which cannot be detected by our current setup. Furthermore, it is still unclear about signaling between astrocytes and pericytes in the capillary level, therefore it is hard to define a specific temporal resolution which is sufficient to study the signaling. At last, in this sentence of the introduction session, we mainly talk about the advantage of 3D imaging rather than 2D. Therefore we decide to delete 'with high spatial and temporal resolutions' from the sentence.

We have specified our spatial and temporal resolution in the method session 5.3.

The paper requires editing of the english grammar and language.

This manuscript has been checked and revised by a native English speaker.

Some figures should be improved.

Detailled remarks and questions are given related to the section number they refer to.

Please see below.

2- surgical procedure
Regarding the protocol I was wondering why you use a cathether in the femoral artery to inject FITC-dextran. A retro orbital injection is faster and more easily performed and much less invasive. As a practical indication the rate of success of the whole procedure should be indicated (e.g Out of 10 mice how many surgery and labelling are expected to provide reliable results)

Thanks for your advice. The catheter for femoral artery is to continuously monitor the blood pressure and arterial blood gases, making sure that the mouse is in the normal physiological state. We believe this is necessary and critical to study the regulation of cerebral blood flow.

The catheter for femoral vein is to deliver anesthesia and inject plasma fluorescent dye. We agree that retro orbital injection is indeed faster and easier. However, making catheter in femoral vein has its own advantages: (1) Continuous i.v. infusion of anesthesia to facilitate a stable physiological state; (2) To avoid administration of anesthesia in the middle of imaging session; (3) FITC-dextran (TRITC-dextran) are metabolized quickly in mice. Continuous i.v. infusion of FITC-dextran (TRITC-dextran) gives us stable image quality during the hours' long imaging session.

The success rate of surgery and labeling are almost 100% in our lab. This requires 3-6 months training and practice with micro-surgery skill and imaging techniques.

3 First two photon imaging session
3.2 please give details on how a typical RFP map looks like and how it is used for in,sertion of the micro-pipette.

We have added representative RFP and GFP map in the figure1 C-D. And the following sentences are added in the method session.

Line 202-207

4.4 Choose one of the marked locations in RFP 'map' as destination. Move the pipette tip to the horizontal plane of the cover glass edge with 30μm distance, roughly pointing towards the destination in the x-y plane.

4.5 Carefully lower the pipette tip in z-axis under the cover glass slip. Then start advancing the glass pipette towards the destination until ~500 μm above the brain surface.

Line 359-363

C. Representative images of Red Fluorescent Protein (RFP) and Green Fluorescent Protein (GFP) using epi-fluorescent illumination are shown. They are used as 'maps' during pipette insertion. Red circles mark locations of 1st order capillaries with >5% vasodilation during whisker pad stimulation. Scale bar: 50 μm.

- what is the GFP epifluorescence imaging mode used for ?

The RFP images display cerebral arteries and arterioles in NG2DsRed mice; while the GFP images provide information of the whole vasculature. We use RFP images as maps because the glass micro-pipette is filled with red fluorescent dye. During the pipette insertion, it is easier to check brain surface, pipette tip with the red epi-fluorescent illumination. By revisiting the GFP and RFP map during pipette insertion and two-photon imaging, it helps us to distinguish veins/venules and arteries/arterioles. Furthermore, if using wild-type mice

instead of NG2DsRed mice in the experiment, GFP image is used as map instead. We have added the following sentence.

Line177-178

GFP 'map' is used to distinguish veins/venules from arteries/arterioles.

Line 186-188

If using wild-type mice instead of NG2DsRed mice in the experiment, GFP image is used as 'map' instead.

3.4 Locations with >5% vasodilation are "marked" on the map ? Please explain what does it consist in

The craniotomy in our preparation covers both part of the whisker barrel cortex and part of somatosensory cortex. The responding area to whisker pad stimulation is usually limited to frontal and lateral region of exposed cortex. Therefore a location with <5% vasodilator response to whisker pad stimulation are considered as being outside of the whisker barrel cortex region.

We have added a few sentences to elaborate this definition in the method session.

Line 184-186

Mark locations of 1st order capillaries with >5% vasodilation during whisker pad stimulation on the RFP 'map' (Figure 1C). Locations with <5% vasodilation are considered as being outside of the whisker barrel cortex region.

5
-TRITC-dextran is mentioned here only in the pape and not in the surgical section. Please detail how it is used (injection protocol , volume etc) Is it used in addition or alternatively to FITC to label blood serum , why ? are there any methodological issues with either of these fluorescent molecules ?

Thanks for this comment. FITC-dextran (green) is used in NG2DsRed mice with red fluorescent pericytes; while the vessel lumen is stained as green. TRITC-dextran (red) is used in virus injection mice with green fluorescent indicator for astrocytic calcium; while the vessel lumen is stained as red.

We have added the following sentences in method session to clarify it.

Line 270-272

To distinguish between astrocytes and vessel lumina, TRITC-dextran instead of FITC-dextran should be used to stain vessel lumina red.

-Please mention explicitly the dimensions (in µm of the hyperstack , heigh can be derievd from the data to be between 32 an 50 µm but the x and y dimensions should be included too)

We have added the following sentences in the method session.

Line 236-237

The total height of each stack may vary from 30 -50 µm, while the x-y plane should roughly cover an area of 60 µm × 40 µm.

6 As this is a protocol paper, if the author could share their implementation of the chan-vese segmentation this would surely be appreciated by the intended readers and potentila users of the present protocol.

Thanks very much for this comment. The analyzing software is completely custom built in MATLAB, which is so far very user-unfriendly unfortunately. And we agree that it would be helpful to provide the MATLAB code so that others can also use Chan-vese segmentation method to analyze vessel diameters. We will work on it, and hopefully publish it online soon.

Results

The two digit precision on the speed of vasodilation /vasoconstriction propagation is probably not significant reagrding the 1s temporal resolution and the 200ms puffing of ATP I would suggest discussing this point. How does the speed of the technique compare to two photon microscopes that use AOD to scan in 2D and 3D. Some of these are now commercially available http://karthala-system.com/wp/aodscope/ or http://femtonics.eu/femto3d-acoustooptic/ and would allow to scan larger volumes with better temporal resolution.

We admit that our method has limitations. We have talked to the specialists who are working with developing AOD system in Femotnics. They claim that with the same spatial resolution and volume size, the AOD system can reach as high as 5 volumes per second. Furthermore, the newly developed light-sheet microscope can also conduct fast volume scanning. Elizabeth Hillman et al. can make 10Hz volume scanning with much larger volume (350×800×100µm) in living mouse brain.

We have added the following sentences in the discussion session.

Line 325-329

In order to make more precise measurement of CVR speed, volume scanning speed should be improved. There are other newly-developed volume-scanning microscopes, for example, two-photon microscope with acoustic optical deflector (AOD) scan as fast as 5 volumes per second with the same spatial resolution and volume size (email communication). Light-sheet microscopes scan even larger volume size (350×800×100µm) with 10Hz volumes per second[2].

Figure 2 Error bars should estimated and be added to figure 2HIJK. how many vessels in how many mice were imaged to produce these data. If it is a single one this should be more clearly stated

Thanks for the comments. This is actually a single recording.

We have added the following sentences in the result session and figure legend.

Line 282-283

In single puff recordings, normalized diameter changes at each ROI were overlaid to compare the responses of different vessel segments (Figure 2E).

Line 285-287

Amplitudes and latencies of dilation and constriction at each ROI in single puff recordings were plotted over the calculated distances from the ROIs to the penetrating arteriole (Figure 2H-K).

Line 374

Measurements are based on a single experiment.

Figure 3 A pipette location should be indicated. B Each trace correspond to an ROI but what do the dotted rectangls on the traces correspond to ?

We have marked the pipette location in Figure 3A. The dotted rectangles correspond to the ATP-induced calcium elevation in each ROI, using criteria > mean + 1.5 × standard deviation. We have added the following sentences in the figure legend.

Line 389-390

B. Multiple ROIs are placed at astrocytic somata and processes. Upon 1 mM ATP puffing, intracellular calcium increased in astrocytic processes but not in somata (dotted boxes), where calcium levels > mean + 1.5 × standard deviation were defined as significant.

Reviewer #3:

Manuscript Summary:
The authors describe an intravital procedure to locally deliver chemical stimuli to brain microvessels while using imaging to capture dynamics including the contraction and dilation of vessels and calcium transients in astrocytes. This is a challenging experiment and it would be useful to have video guidance on how to do the various procedures. With addition of a few clarifications

and specifics, this protocol would be useful.

Major Concerns:

It would be important to clarify that this method provides diameter measurements using axial projections of images, rather than true, 3D measurements of the vessel.

Thanks for the comment. We have added the following sentences in the abstract and introduction session.

Line 41-43

Our method uses fast and repetitive hyperstack two-photon imaging (4D imaging) providing precise diameter measurements by maximal intensity projection of the obtained images.

Line 70-71

We implemented a piezo motor objective in a two-photon microscope to study mouse somatosensory cortex *in vivo*, allowing precise diameter measurements by maximal intensity projections of the obtained images.

Recommend adding a local anesthetic at incision sites such as bupivacaine, 0.125%, or lidocaine.

We use lidocaine in our craniotomy preparation. We have now added the following sentences in the method session.

Line 111-112

Inject 0.5% lidocaine (0.04 mL) subcutaneously under the planned incision and wait for 2 minutes.

Line 119-120

Inject 0.5% lidocaine (0.04 mL) subcutaneously under the planned incision and wait for 2 minutes.

Line 135-137

Inject 0.5% lidocaine (0.04 mL) subcutaneously under the planned incision and wait for 2 minutes.

More details are needed on the hyperstack acquisition. Is the objective continuously moving or is it stopping at each level in z? Please comment on the selection of spacing between images within each stack. 4-5 µm seems like a large step if capillaries are only 4-5 wide, because images could catch just the edges of the vessel resulting in problems in the diameter estimation.

The objective stops at each level in z. The point spread function of our two-photon microscope covers z-axis of ± 5 µm. Therefore even if the plane distance is 4-5 µm, we are still capable of capturing images covering vessel center. We have added the following sentences in the method session.

Line 238-239

The piezo-motor objective stops at each level on the z-axis to acquire the image of each plane.

Please also add some comments about the potential complications of acute surgeries and side effects of inserting the pipette in the discussion.

We have added the following sentences in the discussion session.

Line 330-335

Our mouse preparation also has limitations. Acute mouse surgery may have potential complications. Pain relief medicine and anesthesia may affect the neurovascular responses. Acute neuroinflammation can also be triggered, resulting in microglial activation and leukocytosis in cortical tissues and vessels. Although the tip size of glass micro-pipettes is usually very small (< 1 µm), the insertion procedure and ATP puffing could also potentially trigger microglial migration and embracement of insertion site within 0.5-1 hour post-insertion.

Minor Concerns:

In the abstract, I recommend finding a term that is more specific and descriptive of the purpose of the "puffing" (delivery of drug) rather than "puffing".

We have change the term to 'local ejection'

Title: A 3-dimensional in vivo method to study conducted vascular responses by local ejection using glass micro-pipette in mouse cerebral cortex by two-photon microscope

Summary: We present an optimized local ejection procedure using a glass micro-pipette and a fast two-photon hyperstack imaging method, which allows precise measurement of capillary diameter changes and investigation of its regulation in three dimensions.

Abstract: **Line 41**

Here, we describe a 3D *in vivo* method using two-photon microscopy to study conducted vascular responses elicited by local ejection of ATP with a glass micro-pipette.

There are multiple instances where wording could be improved for clarity.

This manuscript has been checked and revised by a native English speaker.

In the diameter measurement, please clarify how the measurements are oriented in angle, because the angle and location of the diameter measurement is ambiguous for lumpy vessels.

In the Data Processing session, we claim as follow 'Draw a rectangular region of interest (ROI) with width of 3 μm perpendicularly across the vessel longitude (Figure2C).' Furthermore, the width of ROI is very small (3 μm), and we didn't need to take lumpy shape of vessels into consideration. In order to clarify it even clearer, we have added the following sentences in the result session.

Line 280-281

Rectangular ROIs were placed perpendicularly across the vessel to measure vessel diameter change upon ATP puffing (Figure 2C).

Line 369-370

C. Multiple uniquely colored regions of interest (ROIs) placed perpendicularly across the vasculature to measure the vessel diameter.

Please provide more details about the way the agarose is prepared. In PBS, saline? Temperatures?

We prepare the agarose in aCSF. We have changed the following sentences in the method session.

Line 145-147

Cover the exposed cortex with 0.75% agarose gel (type III-A, low EEO; Sigma–Aldrich), dissolved in artificial cerebrospinal fluid (aCSF; pH = 7.4) and cooled to 35°C.

Also, what is the fluid for the dextran and alphachloralose/dextran solutions - saline?

FITC-dextran, TRITC-dextran, alpha-Chloralose are prepared in solution of saline. We have added the following sentences in the method session.

Line 165-166

Both FITC-dextran and α-chloralose are dissolved in 0.9% saline.

A diagram of the pipette angle relative to the mouse might be useful to better describe the coordinate system. Is the pipette coming over the attachment of the headmount?

The pipette holder is mounted on the same stage plate with the mouse. We have changed Figure 1A and figure text.

Line 352-353

The glass micro-pipette holder is mounted on the stage plate.

Please provide drawings/specs for the head holder and mount.

We have changed the diagram in Figure 1A, and add the following sentence in the Method section.

Line 141-142

The metal head bar is 75 mm long and 13.5 mm wide, with round hole of 5 mm diameter in the center.

Editorial comments:

Changes to be made by the author(s) regarding the manuscript:
1. Please note that the editor has formatted the manuscript to match the journal's style. Please retain the same. The updated manuscript is attached and please use this version to incorporate the changes that are requested.

Yes, we have revised the manuscript based on the version you send to us.

2. Please address specific comments marked in the attached manuscript. Please turn on Track Changes to keep track of the changes you make to the manuscript.

We have enabled the Track Changes.

3. Please number the references in order of appearance.

We have revised the references as you required.

4. JoVE cannot publish manuscripts containing commercial language. This includes trademark symbols (™), registered symbols (®), and company names before an instrument or reagent. Please remove all commercial language from your manuscript and use generic terms instead. All commercial products should be sufficiently referenced in the Table of Materials. You may use the generic term followed by "(Table of Materials)" to draw the readers' attention to specific commercial names. Examples of commercial sounding language in your manuscript are: Jackson Laboratory, CWE, Harvard Apparatus, Sigma-Aldrich, Femtonics Ltd, Loctite, etc.

We have revised the manuscript as you required.

5. Please revise the protocol to contain only action items that direct the reader to do something (e.g., "Do this," "Ensure that," etc.). The actions should be described in the imperative tense in complete sentences wherever possible. Avoid usage of phrases such as "could be," "should be," and "would be" throughout the Protocol. Any text that cannot be written in the imperative tense may be added as a "Note." Please include all safety procedures and use of hoods, etc. However, notes should be used sparingly and actions should be described in the imperative tense wherever possible.

We have revised the manuscript as you required.

Please move the discussion about the protocol to the Discussion.

Sorry to ask, where are these specific sentences you are meaning with?

6. The Protocol should be made up almost entirely of discrete steps without large paragraphs of text between sections. Please simplify the Protocol so that individual steps contain only 2-3 actions per step and a maximum of 4 sentences per step. Use sub-steps as necessary.

We have revised the manuscript as you required.

Please move the discussion about the protocol to the Discussion.

Sorry to ask, where are these specific sentences you are meaning with?

7. Surgical procedure: Please specify all surgical instruments used.

We have revised the manuscript as you required.

8. Data processing: Software steps must be more explicitly explained ('click', 'select', etc.). Please add more specific details (e.g. button clicks for software actions, numerical values for settings, etc.).

This is a completely customized software based on mathematical theories and equations. And this software hasn't been rewritten for being shared publically and being user-friendly. We understand that JOVE wants all the details in the method session. But it is unrealistic for us to dedicate several months for software update and give a detailed guideline for buttons to press.

9. After you have made all the recommended changes to your protocol (listed above), please highlight 2.75 pages or less of the Protocol (in cluding headings and spacing) that identifies the essential steps of the protocol for the video, i.e., the steps that should be visualized to tell the most cohesive story of the Protocol.

We have revised the manuscript as you required.

10. Please highlight complete sentences (not parts of sentences). Please ensure that the highlighted part of the step includes at least one action that is written in imperative tense. Please do not highlight any steps describing anesthetization and euthanasia.

We have revised the manuscript as you required.

11. Please include all relevant details that are required to perform the step in the highlighting. For example: If step 2.5 is highlighted for filming and the details of how to perform the step are given in steps 2.5.1 and 2.5.2, then the sub-steps where the details are provided must be highlighted.

We have revised the manuscript as you required.

12. Figure 3: Please include a space between the number and its corresponding unit (10 s).

We have revised the figure as you required.

13. Supplementary videos: Please include a title and a description for each video and place them in the Figure Legends section. Please upload the software used for data analysis to the "Supplemental Code Files" section of your Editorial Manager account.

We have revised the video legend as you required. We suggest these videos are shown in Jove video.

14. Table of Materials: Table of Materials: Please ensure that it has information on all relevant supplies, reagents, equipment and software used, especially those mentioned in the Protocol. Please sort the items in alphabetical order according to the name of material/equipment.

We have revised the table as you required.

15. Please ensure that the references appear as the following: [Lastname, F.I., LastName, F.I., LastName, F.I. Article Title. Source. Volume (Issue), FirstPage – LastPage (YEAR).] For more than 6 authors, list only the first author then et al. See the example below:
Bedford, C.D., Harris, R.N., Howd, R.A., Goff, D.A., Koolpe, G.A. Quaternary salts of 2-[(hydroxyimino)methyl]imidazole. Journal of Medicinal Chemistry. 32 (2), 493-503 (1998).

We have revised the references as you required.

16. References: Please do not abbreviate journal titles. If there are six or more authors, list the first author and then "et al.".

We have revised the references as you required.