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Cerebral vascular and hemodynamic imaging with 2-photon microscopy

by

Eric I-Ju Lee

THESIS

Submitted in partial satisfaction of the requirements for the degree of

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in

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UNIVERSITY OF CALIFORNIA, SAN FRANCISCO

Cerebral vascular and hemodynamic imaging with two-photon microscopy

Eric I-Ju Lee

Abstract

A functional brain vascular supply is crucial for delivery of life-essential nutrients and removal of metabolites. Abnormal vascular development and hemodynamics can result in pathologies of many vascular diseases. The enlarged high-flow blood vessels that shunt blood from arteries to veins can cause arteriovenous malformation (AVM) and it can lead to life-threatening ruptures in the brain. The ability to correlate the relationship between blood flow with vascular structure at cell levels in living animals would foster our knowledge of the disease. Conventional wide-field microscopy is powerful in imaging at cell levels; however, light penetration depth is the limitation in deep tissue imaging. Near-infrared fluorescence imaging system can achieve deep tissue imaging but the low spatial resolution makes it difficult to map vascular structure and blood flow. In this research, we use two-photon laser scanning microscopy that can achieve deep tissue imaging and high spatial resolution to do cerebral vascular imaging in the genetic mutant mice that showing the phenotype of AVM. By using the line-scan imaging and 3D scan, we are able to analyze the blood velocity and diameter of the blood vessel that give the dynamic information of blood flow and vascular structure. We hope the findings through "5D" two-photon microscopy imaging that include high spatial vascular structure (3D) and blood velocity (4th dimension) over period of time (5th dimension) can improve insights for the mechanism of AVM formation in the brain.

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1. INTRODUCTION

The ability to analyze the functional vascular supply in the brain is important in studying vascular development, physiology and pathology of vascular diseases. A normal brain vascular supply is crucial for delivery of oxygen, nutrients and signaling molecules to cells and for removal of metabolites[1]. Abnormal vascular development can result in many pathologies of many diseases and lead to atherogenesis, arteriogenesis and vascular remodeling[2, 3]. The importance of hemodynamics plays a role to help us understanding the shaping of blood vessels and the origin of vascular disease[4, 5]. The enlarged high-flow blood vessels that shunt blood from arteries to veins is the pathology of a wide range of vascular diseases such as arteriovenous malformations (AVMs)[6]. These devastating condition can form anywhere in the body and can lead to life-threatening ruptures. The ability to correlate blood flow with vessel structure and molecular-level dynamics in living animals would improve our understanding of vascular development and disease.

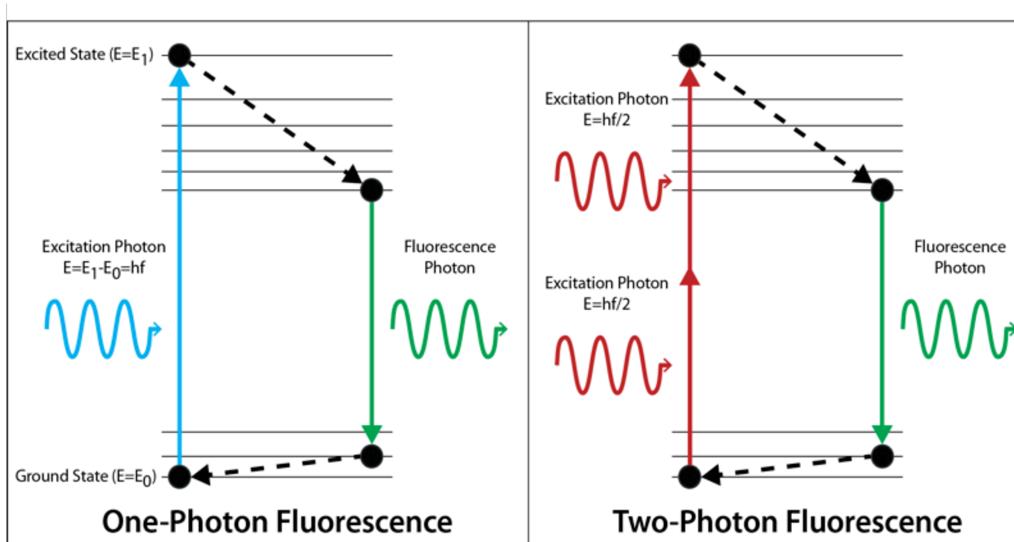
Conventional wide-field imaging methods such as fluorescence microscopy is a powerful tool to image down to cell levels in biological vascular research[7]. However, the technique is limited by light penetration depth in deep tissue imaging. Light scattering and absorption by common biological molecules within visible light range which is commonly used in fluorescence microscopy are the main reasons that limit the penetration depth[8]. In vivo near-infrared (NIR) fluorescence imaging system that uses light range around 700-1000

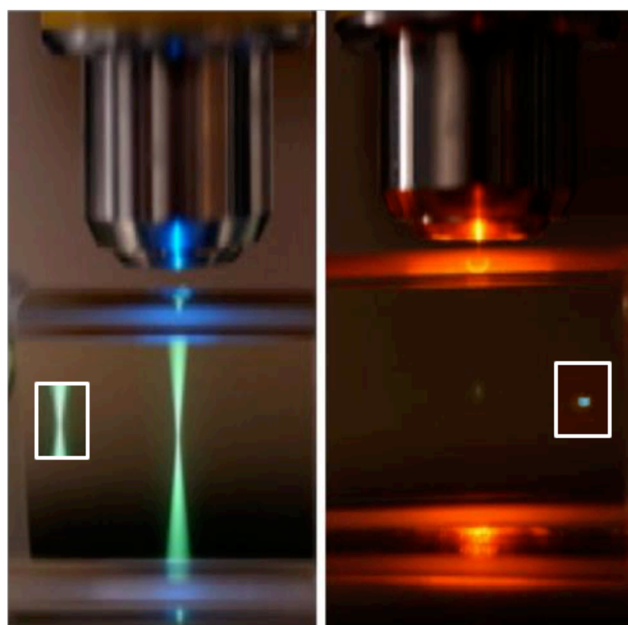
nm has better light penetration in tissue and has been reported to be used in small animal imaging of vascular mapping and tissue perfusion[9]; however, the spatial resolution of the system is around 1 mm that is difficult to resolve vascular development and flow dynamics at cell levels. For biological vascular research, the technique to image and quantify blood velocities in individual vessels deep in living tissue with high resolution to cell level is needed.

Two-photon laser scanning microscopy (TPLSM) can play a complementary role and has long been the tool for studying hemodynamics and vessel structure at high resolution in mice and rats[1, 10]. Contrary to traditional fluorescent microscopy, which use visible light as excitation source and generate contrast from single photon excitation, TPLSM use near-infrared light as the excitation source to generate signal from two photon excitation. The energies of the two photons that absorbed by the molecule at the same time (within ~ 0.5 fs) can promote the molecule to excited state, and then undergoes normal fluorescence emission process (Scheme 1). The efficiency of two-photon absorption relies on spatial and temporal distribution of excitation light. The spatial distribution of light can be achieved by focusing the beam using high numerical aperture (NA) objective lens, while the temporal distribution of light requires the use of ultra-short pulsed laser with high peak intensities. The advantages of TPLSM are that the use of NIR light allows deep penetration to hundreds of micrometer in the tissue because of less light scattering and absorption, and the lack of out-of-focus

excitation reduce photodamage to the tissue thus make long-term live time imaging plausible[8].

In this research, we use mice as our animal model to do cerebral vascular imaging by TPLSM. By administering of fluorescent agent into the blood vessel, the labeling blood plasma is used to form the map of vascular structure as well as quantify the movement of red blood cells (RBCs). The line-scan in TPLSM system has been used to follow RBCs motion in a target vessel. When blood plasma is labeling with fluorescent agent, the individual RBC appears dark spot in the image and these dark spots form streaks in the space-time scanning data (x-t image)[11]. The speed of the RBCs can be calculated by getting the inverse value of the slope in each streak. To understand the correlation of blood flow with vessel structure to AVM, we examined a mouse model of AVM by knock-out Alk1 gene in the brain from early post natal birth stage, illustrating the progression of the disease using two-photon excited fluorescence imaging, which can acquire high resolution of vascular structure and measure blood velocity over period of time. Our findings can provide insights for the mechanism of AVM formation in the brain.





Scheme 1. Principle of two-photon excitation versus single-photon excitation

2. MATERIALS AND METHODS

2.1 Mice preparation

This study was performed with NIH regulations and the Institutional Animal Care and Use Committee at the University of California San Francisco. The protocol was approved by the Institutional Animal Care and Use Committee (IACUC) at UCSF (Approval Number: AN165932-02). Mutant mice (Alk1-/-) were generated to develop AVM in the brain. Wild type mice (Alk1+/+ or Alk1+/-) were used as control group to study normal cerebral vasculature. To achieve chronic *in vivo* brain vascular imaging, a small craniotomy was performed over the cortex of the mice as described[12]. The cortex was bathed in artificial cerebral spinal fluid, covered with a 5 mm coverglass (World Precision Instruments), and sealed with dental acrylic (Lang Dental) material to provide optical access to the brain. Before *in vivo* brain vascular imaging, the mice with a cranial window were anesthetized with 0.5-2% isoflurane in 2 L/min of oxygen and warm-fixed on a thermal blanket (Harvard Apparatus) (Figure 1A). All animals were treated in accordance with the guidelines of the University of California San Francisco IACUC.

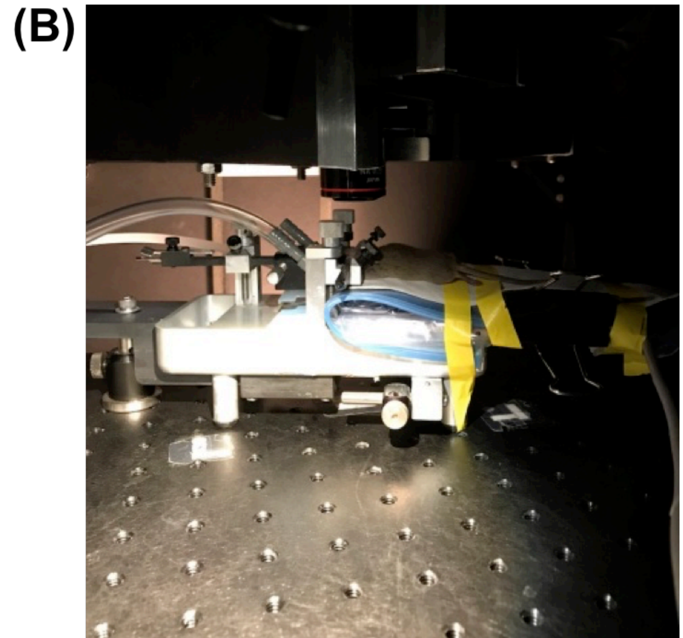


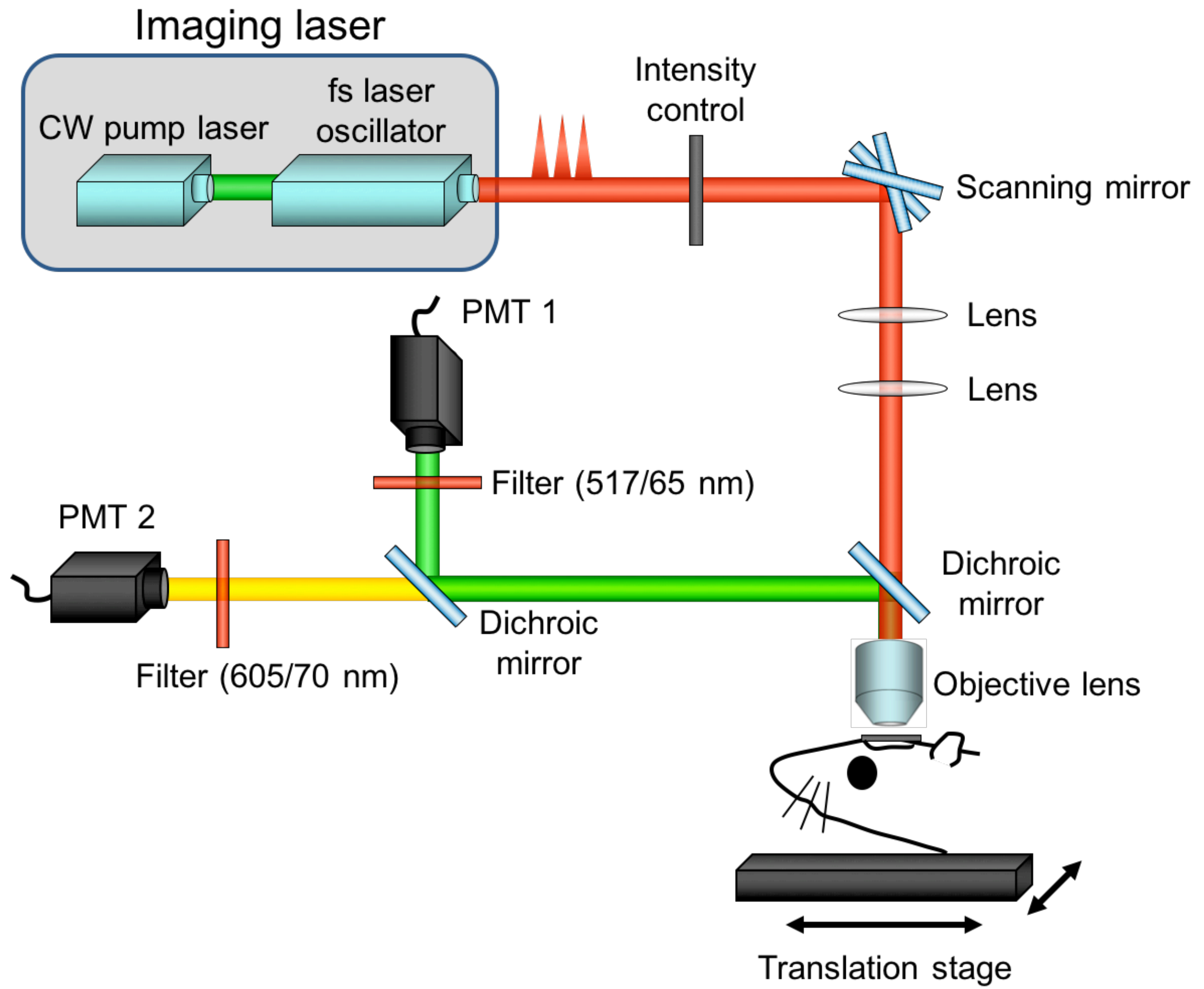
Figure 1. Preparation of mice for *in vivo* imaging

(A) An anesthetized mouse shows a cranial window at the right parietal cortex. (B) The anesthetized mouse is warmed fixed with a stereotaxic stage under the two-photon microscopy.

2.2 Setup of two-photon microscopy and *in vivo* imaging

To image the blood vessel, we inject 0.1 ml of a 1% (wt/vol) solution of 2000 KDa FITC-dextran (Sigma) through the retro-orbital vein of the mice for labeling the blood plasma. The head with a cranial window was immobilized with a stereotaxic stage (myNeuroLab.com) for imaging (Figure 1B). Fluorescence image of the mouse brain was performed by a home-built TPLSM system (Scheme 2). Two-dimensional images and line-scan data were acquired by the TPLSM system equipped with a long working distance, 1.0 numerical aperture (NA), 20X, water-immersion objective (Zeiss). FITC fluorescence was excited using a Titanium:Sapphire laser oscillator (Mai Tai HP, Newport Spectra-Physics) that was pumped by a continuous wave diode laser (Millennia, Newport Spectra-Physics) and passed through a dispersion compensator (DeepSee, Newport Spectra-Physics) with low-energy 100 femtosecond laser pulses at 80 MHz, centered at 800 nm. Intensity of the laser was controlled by rotation of a $\lambda/2$ wave-plate to a polarizer and using the transmitted beam. The laser beam was scanned by galvanometric mirrors (Cambridge Technology) and then conducted to a microscope platform. The two-photon excited fluorescence was collected by the objective, reflected by a dichroic mirror (700 nm long-pass, Chroma), splitting into green and red channels with a secondary dichroic mirror (560 nm long-pass, Chroma) and relayed to photomultiplier tubes (H7422P-40MOD, Hamamatsu). Fluorescence of FITC-dextran was spectrally collected by placing additional band-pass filter (517/65 nm, Chroma) before the

PMT. In this work, all consecutive images were obtained with a pixel density of 1024 × 1024 and at 2 μm axial spacing for stack images. We used line scans through target blood vessels with a maximum scan rate of 1.85 kHz to quantify flow velocity.



Scheme 2. Schematic of the home-built two photon laser scanning microscopy

2.3 Analyze flow velocity and vessel diameter

The scheme illustration of analyzing flow velocity is described in Figure 2A. The high-molecular weight FITC-dextran was injected into the vascular to enable imaging the perfused blood vessel. The individual RBC did not absorb the fluorescent agent and therefore appeared black dot in the image by using TPLSM. To do line-scan imaging, we selected a Region of Interest (ROI) of the target vessel and recorded the image of central axis for a period of time (~50 s). The displacement of RBCs between line-scans were determined by the cross-correlation and the data processing algorithm was built with collaborative lab at Cornell University[13]. The shift in pixels (Δx) was processed by the algorithm and the velocity was calculated by $v = \Delta x / \Delta t$, where Δt is the time between each line-scan.

The principle of analyzing vessel diameter is shown in Figure 2B. The cerebral vasculature was imaged through the cranial window over the right parietal cortex and the blood plasma labeled with FITC-dextran appeared bright fluorescence under the microscope. The image stacks of cerebral vascular were acquired with 2 μm steps along z-axis and overlaid with maximum projection. To analyze the diameter of the blood vessel, we select a target vessel from the overlaid image and the data analyzing algorithm was written with Matlab (MathWorks). The cross-section intensity was extracted by dragging a straight line perpendicular to the target vessel. The diameter of the blood vessel can be calculated from the full width at half maximum (FWHM) from the cross-section intensity.

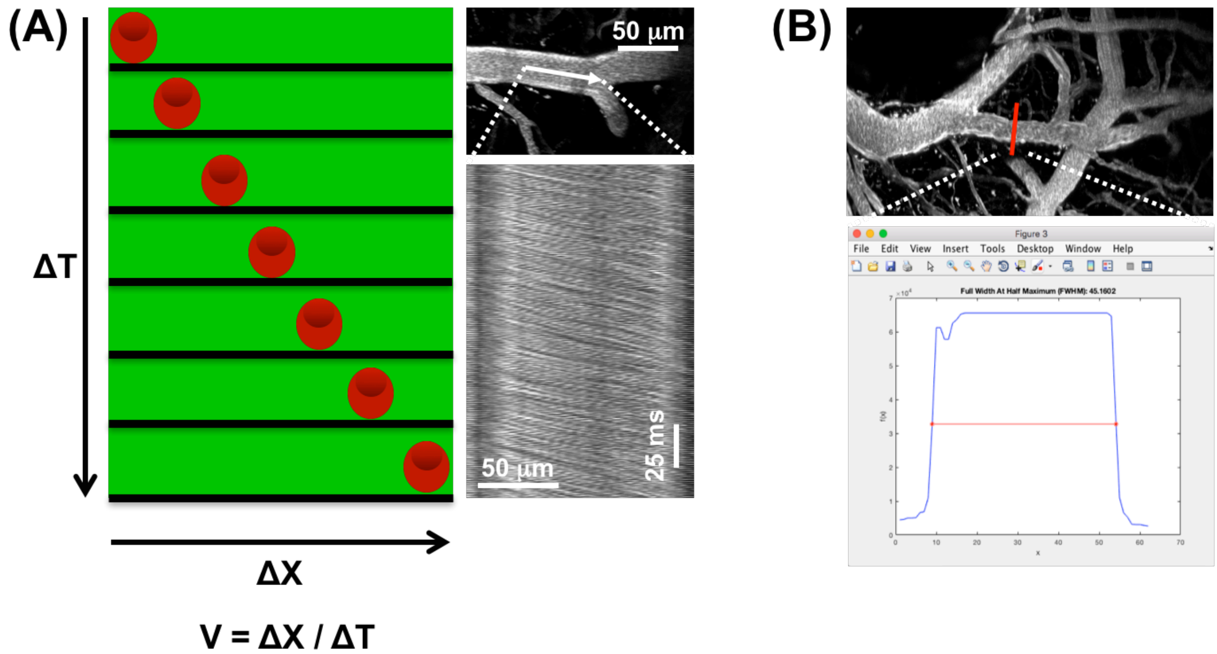


Figure 2. Analysis of flow velocity and lumen diameter of target blood vessel

(A) Schematic of line-scan image and the principle of calculating flow velocity. *In vivo* two-photon fluorescence image shows the center line of artery for flow analysis and line-scan demonstrates a space-time image with time increasing from top to bottom. Single RBC appears as each dark streak as it moves along the scan path. (B) *In vivo* two-photon fluorescence image shows the target blood vessel for diameter measurement. The cross-sectional intensity of the vessel shows a wave-form graph. The diameter is calculated from FWHM of cross-sectional data.

3. RESULTS

3.1 Demonstration of data processing of diameter and flow velocity

To demonstrate the data processing of vessel diameter and flow velocity, consecutive stack images of cerebral vasculature and line-scan of target blood vessel was performed in the brain of wild type mice by using two-photon microscopy. Typical cerebral vasculature in the parietal cortex of a mice is shown in Figure 3A, containing arteries, capillaries and veins that form a complicated structure providing functional supply to the brain. The diameter of artery measured in the ROI was 28 μm, 7 μm in the capillary and 23.6 μm in the vein (Figure 3B). The analysis of line-scan image showed pulsatile flow that made it easy to see the rapid change in velocity due to the heartbeat and the averaged flow velocity measured in the same artery was 6.6 ± 0.6 mm /s, 2.3 ± 0.2 mm / s in the capillary and 1.4 ± 0.1 mm / s in the vein (Figure 3C).

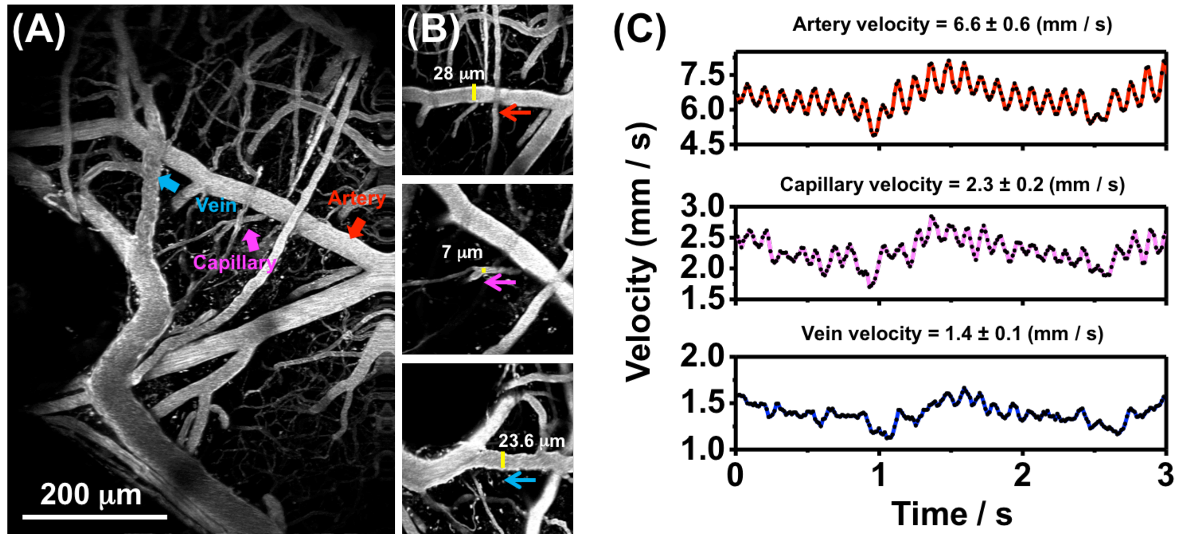


Figure 3. Two-photon fluorescence imaging of cortical brain vessels

(A) Cerebral vascular of cortical brain and plasma was labeled by injection of FITC-dextran. Color-coded arrows indicate different type of blood vessel. Red: artery, Purple: capillary and Blue: vein. (B) Images of artery, capillary and vein that flow velocity was measured by line-scan along the center line of the vessel. Diameters of vessels were measured trans-axially. (C) Velocity of each target vessel calculated from line-scan image.

3.2 Demonstration of time-lapse two-photon fluorescence imaging of cortical cerebral vessels in the genetic mutant mice showing AVM

To determine the formation of AVMs, longitudinal time-lapse imaging in the brain of the Alk1-/- mice was performed. Cranial windows were implanted at the right parietal cortex of mice at 10 post natal days (p10) and the vessel diameter and flow velocity were recorded over time by using two-photon microscopy. We measured the lumen diameter of capillaries that connecting arteries and veins. We defined AV shunting if the diameter of vessels increases over 15 μm because this diameter was not found in the AV connection of control mice. According to the time-lapse images taken by two-photon microscopy, we found the capillary-like vessels started to enlarge and developed AV shunt in mutant mice at ~p18 (Figure 4B). Of all the vessels measured, 18 out of 26 vessels (69.2 %) in 5 mutant mice grew larger than 15 μm, whereas no significant change in the 9 vessels of 3 control mice (Figure 4A).

We examined the flow velocity of AV shunts at the beginning of the development. Using two-photon microscopy imaging, we were able to analyze the red blood cell velocity and the diameter of AV connection simultaneously. The correlation of flow velocity and the diameter of AV connection vessels is demonstrated in Figure 5. The AV connection vessels enlarged with the initial increase in flow velocity (Figure 6B1, 6/26 vessels in 5 mice). Some capillary-like vessels enlarged without a significant increase in flow velocity (Figure 6B2, 12/26 vessels in 5 mice) while some appeared an increase in neither diameter nor velocity

(Figure 6B3, 8/26 vessels in 5 mice). The control mice showed no significant increase in neither diameter nor velocity (9 vessels in 3 mice, Figure 6A). The data are summarized in Figure 6C. Currently, our result shows that flow velocity in the AV connections grew into AV shunt is capillary-like in the beginning but not mainly abnormally elevated when the vessels enlarge.

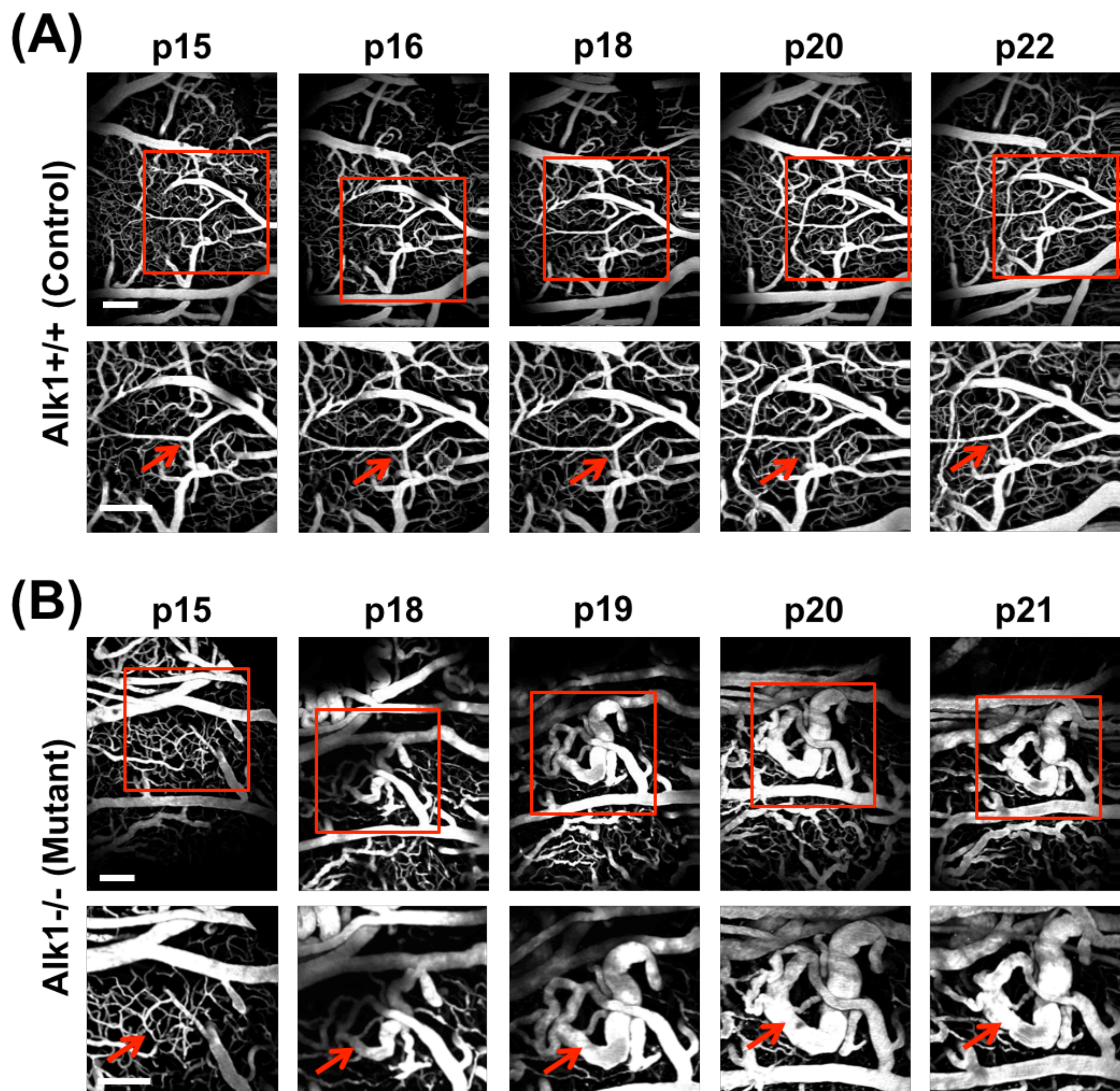


Figure 4. Time-lapse two-photon fluorescence imaging of cortical brain vessels in Alk1-/- mutant mice

The bottom row images showed the red square window images from top row. Scale bar = 100 μm (A) The control mice showed no significant enlargement of capillary-like vessels compared with mutant mice. (B) Mutant mice developed AV shunts through enlargement of the capillary-like vessels (red arrows) between p18 and p21.

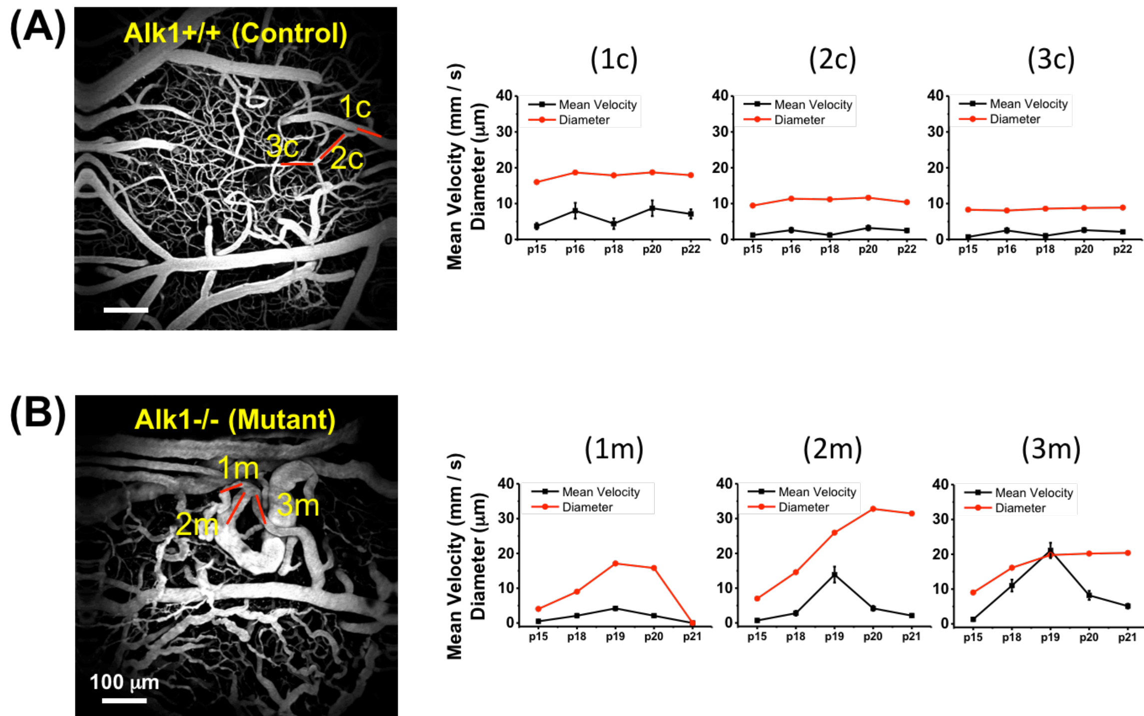
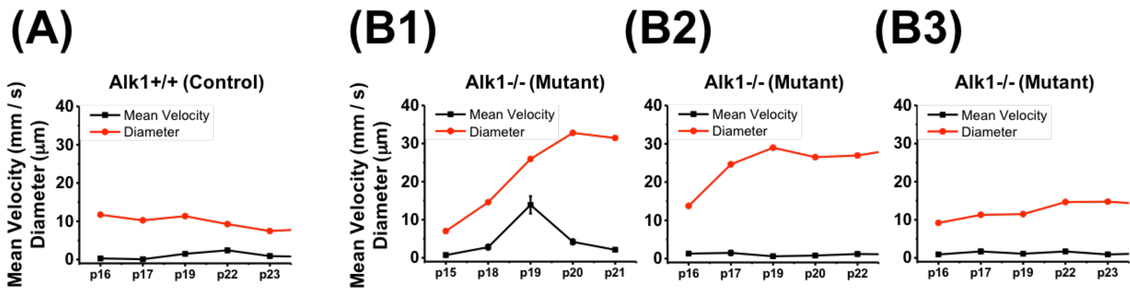


Figure 5. Correlation of flow velocity and lumen diameter of target blood vessels

(A) Flow velocities (black) and vessel diameters (red) measured overtime in the AV shunts of control mice. (B) Mutant



(C)

Vessels connecting arteries and veins	Numbers
Vessels below 15 μm @ p15	26
Velocity + Diameter increase	6
Diameter increase only	12
No significant change	8

Figure 6. Data summarization

(A) One type of correlation between flow velocities (black) and vessel diameters (red) measured overtime in the AV shunts of control mice. (B) Three types of correlation measured overtime in the AV shunts of mutant mice. (C) Summarized data

4. DISCUSSION AND FUTURE WORK

In this research, TPLSM system is a powerful imaging tool to study vascular biology in whole tissue and living organism with high resolution. By using the line-scan image and 3-D image stacks, we demonstrated the change of hemodynamics and the cerebral vascular structure in the mutant mice that showing the phenotype of AVM; however, some points remain for further confirmation in the future. First, only 23% of the vessels showed the enlargement of lumen diameter with the increase in flow velocity. This result contradicted to the typical high-flow shunting in the development of AVM. More experimental design is needed to prove the mechanism. Second, demonstration of the proliferation and migration of endothelium cell during AVM development can facilitate the understanding the mechanism of the disease. Although it has been reported that the deficiency of Alk1 gene altered the movement of endothelium cells but not altered proliferation of endothelium cells in zebrafish[14], the cellular dynamics related with blood flow during Alk1 deficiency induced AVM development in mice is unknown. We propose a future work to investigate the dynamics of endothelium cells during development of AVM in Alk1 deficiency mutant mice. We hope our findings can provide insights for the mechanism of Alk1 deficiency induced AVM formation in the brain.

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