New Method to Quantify Angiogenesis *in vivo* Using Multi-photon Imaging

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Introduction

Efforts to understand the basic mechanisms of angiogenesis, that is, the formation of new blood vessels from existing vasculature, have been limited by the methods that are currently used to measure vessel growth. Although *in vivo* assays provide the best environment in which to track angiogenesis, inherent difficulties in obtaining reproducible data limit the power of this approach. Limitations include: environmental variations between experimental animals, induction of inflammatory responses by surgical methods, and labor-intensive blood vessel quantification procedures. A better assay would measure vessel growth in one animal at multiple time points and would focus on minimization of artifacts induced by experimental manipulation.

Angiogenesis is essential for normal embryonic development, wound healing, and post-ischemic tissue repair; it is also associated with such pathological conditions as cancer, diabetes, and psoriasis. If we can develop means to control this process, we acquire important therapeutic tools to combat a number of diseases [1]. Thus, angiogenic research is an active area of investigation in both preclinical and clinical settings.

Because virtually all adult tissues maintain a well-developed vasculature, it is difficult to measure angiogenesis *in vivo*. Several strategies have been developed that either follow vessel growth into an avascular tissue of an experimental animal, as in the corneal implant assay [2], or that follow vessel growth into an avascular substrate, as in the Matrigel implant assay [3]. Although these approaches have enabled important progress in angiogenic research, they are limited by the associated high experimental variance and undesirable host responses, such as inflammation. Our goal in this study was to develop a method that would minimize the limitations of the conventional *in vivo* angiogenesis assay while permitting maximal quantitation. To this end we have developed a method that uses multi-photon microscopy to quantify subdermal vasculature in mice over a several-day time span.

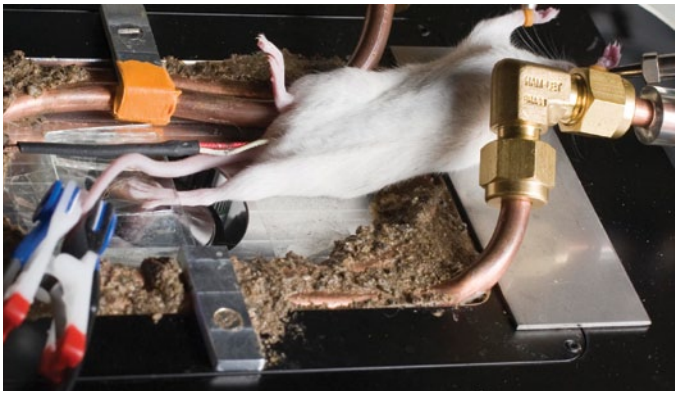

Figure 1: Demonstration of an anesthetized mouse on the microscope stage, undergoing vessel imaging. The sensor for the MouseOx monitoring system, as well as the rectal temperature probe, are visible.

Methods

Animals. All animals used in this study were handled in accordance with the guidelines of the Wadsworth Center's Institutional Animal Care and Use Committee. FVB/N-Tg (TIE2-lacZ) or A/J mice were used in all experiments. Mice were anesthetized either by exposure to the inhalation agent isoflurane or through an intraperitoneal injection of 8 mg/mL ketamine combined with 1 mg/mL xylazine. Once a mouse was anesthetized, 15 µL of 2X concentration BD Matrigel™ (BD Biosciences) containing 100 ng/mL rhVEGF165, 300 ng/mL bFGF (R&D Systems Inc.), and 25 ng/mL heparin was injected into the hind leg, proximal to the hind paw, via an intradermal injection. Control mice were injected with 15 µL of only the 2X concentration BD Matrigel™ alone. Additionally, to evaluate whether BD Matrigel™ by itself induces vessel growth, we injected another group of control mice with 100 ng/mL rhVEGF165, 300 ng/mL bFGF, and 25 ng/mL heparin in 1X phosphate-buffered saline.

Once injections had been administered, mice were returned to their cages and allowed to recover for approximately 24 hours before imaging. We chose to evaluate the anterior glaborous skin of the hind leg proximal to the subtalar joint because this region is easily isolated on the microscope stage and does not require hair removal prior to imaging. Immediately prior to imaging, anesthetized mice were given a 100ul lateral tail vein injection of 70-kDa FITC-conjugated dextran (Sigma Aldrich) in sterile 200mM dextrose in 0.9% NaCl. Vital signs, including pulse, respiration, and oxygen saturation were monitored with the MouseOx system (Starr Life Science Corp.) throughout all procedures.

Imaging. Individual mice were restrained and imaged on a custom-machined microscope stage as shown in Figure 1. The temperature of each mouse was maintained by flowing 37°C water through tubing embedded on the stage. Mice were imaged on day 1, 6, and 9 post injection with a Leica SP5 confocal equipped for spectral and multi-photon imaging (Mai Tai laser, Spectra Physics). The MP laser was tuned to 820 nm to image the FITC-labeled dextran and 810 nm to image expressed GFP. Non-descan detectors were used to collect all image data. These

https://doi.org/10.1017/S1551929509000339 Published online by Cambridge University Press

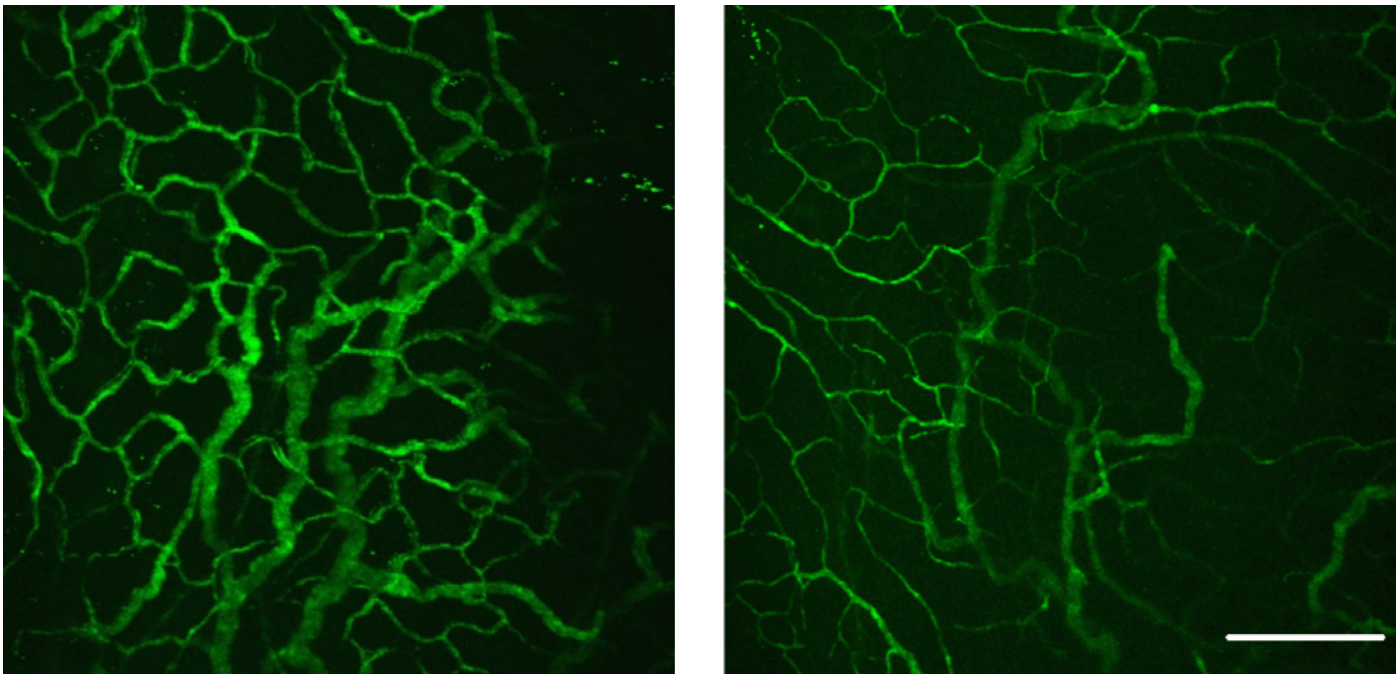

Figure 2: Comparison of 2D projections of vessels (processed in 3D—see text) imaged from mice anesthetized with either isoflurane (left image) or ketamine (right image). The apparent larger vessel diameter in the isoflurane image is due to movement during the imaging, which could not be fully compensated with image processing. Scale bar = 100 μm

detectors are located as close to the objective lens as possible and outside the confocal scan head, thereby maximizing the signal at the lowest possible photon dose [4]. A 20X (0.7 NA) water objective lens was used to collect all images. For spectral emission determination, an internal PMT was used. The spectra were collected from 450 to 600 nm with 5 nm resolution. To capture three-dimensional (3D) information, Z stacks (1.2 μm/slice) were collected. Mice anesthetized with ketamine/xylazine had significantly less movement and muscle twitching than did mice anesthetized with isoflurane (Figure 2). Although it is possible to reduce the effects of such movement in the resulting stack projection via 3D digital image processing, less image processing is always more desirable. Additionally, the isoflurane caused a higher background level in the surrounding interstitial spaces, especially after the second and third injections of the dextran. Because the emission spectrum of the background structures was very similar to the spectrum for the vessels, we suspect that dextran/FITC leaks out of the vessels over time (Figure 3).

Image processing and analysis. Image stacks were processed with a 3D Gaussian filter (ImagePro, Media Cybernetics) to reduce noise and to smooth motion effects introduced into individual sections by the animal's breathing and heartbeat. Image stacks collected from ketamine/xylazine-anesthetized mice were deconvolved to further improve the signal-to-noise ratio and to facilitate tracing of the vasculature

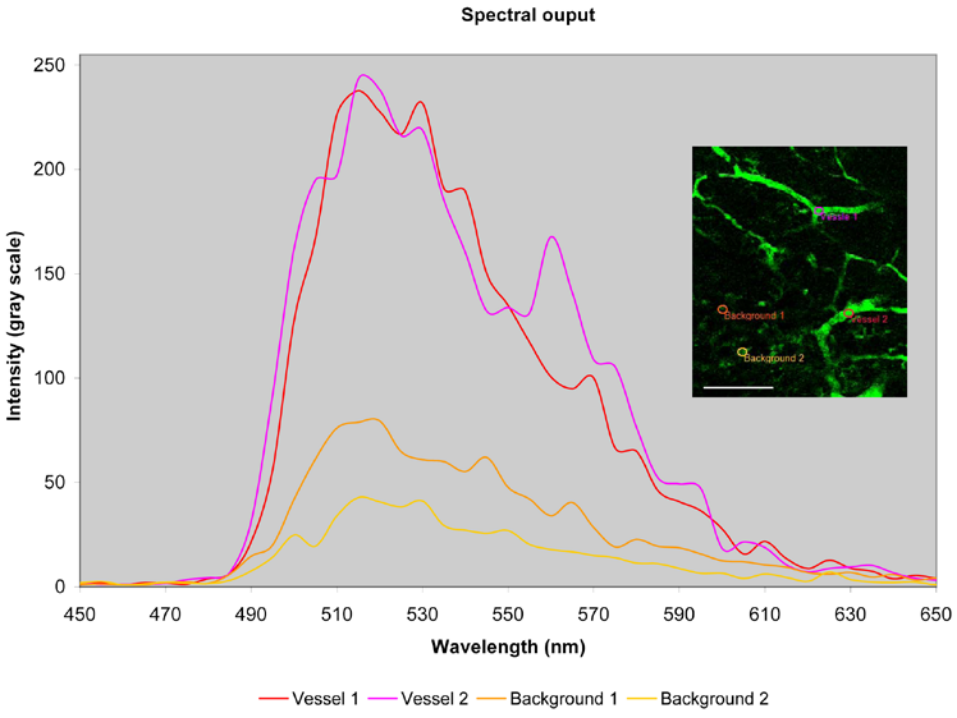

Figure 3: Spectra emitted from labeled vessels (FITC/dextran) and from the interstitial spaces. Inset photo shows the sites of collection of the spectra. The vessels and the interstitial spaces have similar spectral output indicating the presences of FITC/dextran in both compartments. Scale bar = 100 μm

(Figure 4) (Autoquant, Media Cybernetics). Vessel segments were traced on composite images between branch points with ImageJ using the neuron J plugin [5]. For quantification of measurements, the total number of branch points (as a surrogate for total individual segments), total vessel length, and average vessel length were determined.

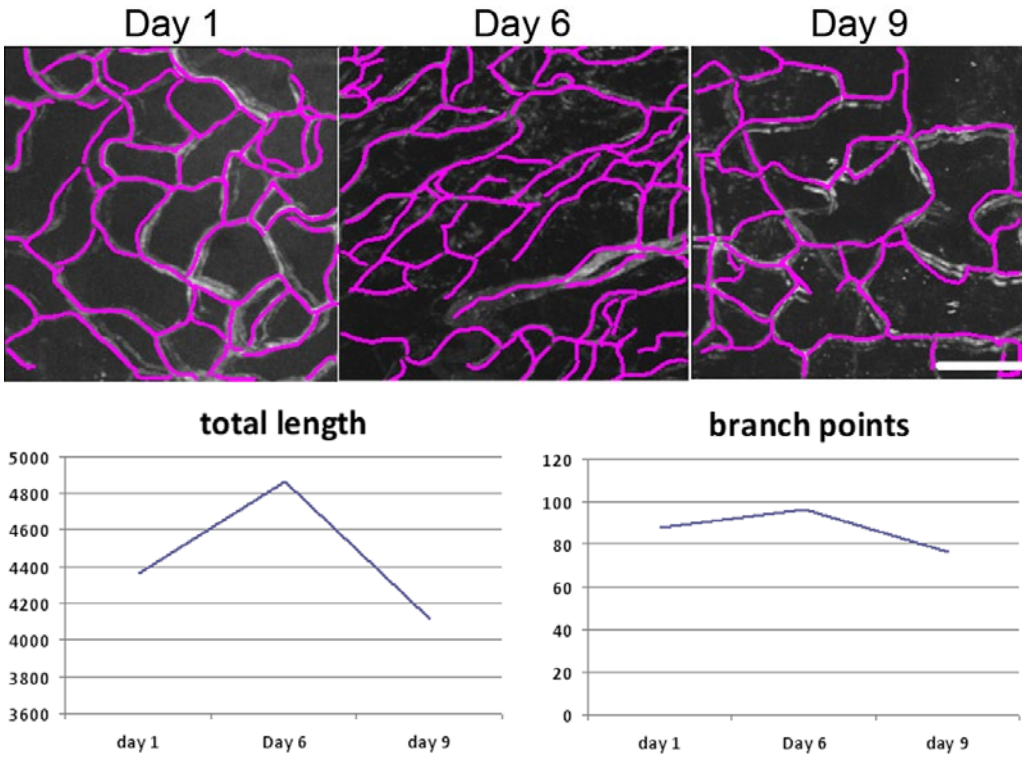

Figure 4: (A) Representative composite image of vessels at Day 1, Day 6, and Day 9 visualized with FITC Dextran. Tracings of vessels performed in Neuron J. (C) Graph showing average vessel branching and total length on days 1 through 9. Scale bar = 100 μm

Results

Using multi-photon intravital imaging, we demonstrate that it is possible to repeatedly image, via a Z-series, the same area over a period of days or even weeks. Our data demonstrate that significant differences in branching and total vessel growth can be obtained from minimal numbers of research animals. Maximal increases in total vessel length were observed on day 6 with regression of vessel growth by day 9. The multi-photon excitation, among all imaging modalities, produces the lowest photon dose in the sample. This dose minimization enables us to follow angiogenesis in the same animal over several days, greatly minimizing sample-to-sample variation. Additionally, because we use a bolus of Matrigel that is lower than the conventional Matrigel plug assays [6] we can make efficient use of novel angiogenic compounds when reagent quantities are limiting.

Analysis of the same region in a given animal over several time points resulted in the appearance of small deposits of FITC-labeled material outside of the vessels. This may be a result of the dose of VEGF (known to induce vessel leakage) used in these preliminary studies [7]. Ongoing studies are exploring this issue.

Initially, when compared to the mixture of ketamine and xylazine, isoflurane appeared to be the superior anesthesia for our purposes. It has a quick onset time and provides prolonged duration of anesthesia. In addition, unlike ketamine, isoflurane is not a controlled substance, so it is readily available and convenient to use. However, the continued administration of isoflurane while the animal is restrained on the microscope is problematic because this agent may interfere with vascular tone, affecting the image collection [8].

Our ongoing work will focus on three central issues: determination of the best dose response and time series for measurement of blood vessel growth, development of semi-automated vessel quantification procedures that minimize observational bias, and expansion of our animal model to include the evaluation of more complex aspects of angiogenesis, namely the incorporation of circulating endothelial progenitor cells into neovessels.

Acknowledgments

This work was supported by the Wadsworth Center Advanced Light Microscopy & Image Analysis Core Facility and a grant from the NIAMS grant # R01AR054828 and from NCRR grant # 1S10RR023451. MT

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