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# Automated image analysis programs for the quantification of microvascular network characteristics

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ABSTRACT

The majority of reports in which microvascular network properties are quantified rely on manual measurements, which are time consuming to collect and somewhat subjective. Despite some progress in creating automated image analysis techniques, the parameters measured by these methods are limited. For example, no automated system has yet been able to measure support cell recruitment, which is an important indicator of microvascular maturity. Microvessel alignment is another parameter that existing programs have not measured, despite a strong dependence of performance on alignment in some tissues. Here we present two image analysis programs, a semi-automated program that analyzes cross sections of microvascular networks and a fully automated program that analyzes images of whole mount preparations. Both programs quantify standard characteristics as well as support cell recruitment and microvascular network alignment, and were highly accurate in comparison to manual measurements for engineered tissues containing self-assembled microvessels.

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## 1. Introduction

The quantification of microvascular network characteristics is of interest to a wide variety of researchers. Changes in the microvasculature have been implicated in a variety of disease processes, from neurological disorders to cancer [1,2]. In addition, the development of microvascular networks *in vitro* has been pursued by many for either tissue engineering purposes or as a model for the study of endothelial cell (EC) biology. In all of these areas the quantification of microvessel characteristics is of critical importance in order to statistically differentiate between different treatments or experimental conditions.

A commonly used metric is capillary density, which actually comprises several different metrics. One is quantified from tissue cross sections and reported as capillaries/mm$^2$ [3–5]. A second, also reported as capillaries/mm$^2$, is quantified via nailfold capillaroscopy, in which a finger is viewed under light microscopy and the skin capillaries counted [6,7]. Although these quantification methods are reported with the same units, they are quite different and should not be compared directly. Both methods typically rely on manual counting, which is tedious and can introduce bias.

Another parameter commonly quantified is the network length per image area [8,9]. This parameter is typically used when the entire microvascular network can be viewed, for example in a whole mount tissue preparation or a dorsal window chamber. However, this method also relies on manual measurement of the lengths of the capillaries, which is time consuming. A high degree of subjectivity is also introduced, as the image often contains capillaries that are varying distances from the focal plane, and the observer must decide which capillaries should be included in the measurement.

The introduction of subjectivity into measurements is extremely problematic in the analysis of engineered microvascular networks, as the observer must first define what qualifies as a capillary. In cross section, not all EC structures contain lumens, and some structures contain multiple lumens either because it was sectioned near a bifurcation point or because the several small lumens have not yet matured into a single lumen. In whole mount preparations, microvessels often have abnormal morphology that must be measured accurately or endothelial cell debris that must be eliminated from measurements. These conditions increase the variability in both inter- and intra-observer measurements.

Some work has been done to automate the detection and counting of capillaries. Both Ranefall et al. and Kim et al. reported

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methods for automated capillary counting in immunostained sections imaged under light microscopy [10,11]. Although these methods were shown to be relatively accurate, they counted capillaries by counting positive EC staining rather than the lumens themselves. This poses a problem for use with engineered microvessels, in which a positively stained object may correspond to zero or several lumens. Additionally, characteristics such as lumen size or shape, which are also of importance in microvascular networks both *in vitro* and *in vivo*, were not quantified. The alignment of capillaries was also not quantified, which would be present in longitudinal sections of anisotropic native or engineered tissues such as muscle. Finally, the use of immunohistochemistry rather than immunofluorescence limits the possibility of identifying additional cell types, such as support cells, in the same section, because it would be difficult to distinguish the different cell types in the light microscopy image. Without identifying both cell types in the same section, it would be impossible to accurately quantify parameters such as the recruitment level of the support cells, which is an important indicator of microvessel maturity.

Much more work has examined automated methods for quantifying microvessel length. Methods have been developed for analyzing both brightfield and fluorescent images, both *in vitro* and *in vivo* networks, and both single images and z-stacks for 3D reconstruction [12–19]. Each method has its advantages and disadvantages; some require the input of a binary image, which is at times non-trivial to obtain [17], some require perfusion of the network for imaging [13,16], which cannot always be done for engineered microvessels, and some require extensive serial sections to create a 3D image of the network [14]. None of these methods, however, address the quantification of mural cell recruitment or network anisotropy, which are important parameters to assess both *in vitro* and *in vivo*, as they provide information about the maturity and alignment of the network, respectively.

The methods outlined here for analysis of cross sections and whole mount preparations of engineered microvessels (Fig. 1) address some of the limitations noted above. The semi-automated cross section algorithm detected lumens within fluorescent images based on dark areas surrounded by EC staining which, once identified, can be counted and measured as desired. The fully automated whole mount program quantifies microvessel length as well as bifurcation points. Both programs also share two major improvements over others: the inclusion of methods to measure support cell recruitment to the network and network anisotropy. These inclusions provide additional quantitative information regarding microvascular maturity and alignment, which are useful in many types of microvascular studies. Although user input is required to define the lumens in the cross section algorithm, use of the algorithm saves time over fully manual processes and provides unbiased information about the lumens identified (e.g. lumen area, support cell recruitment, and microvessel anisotropy). The particular images used here to demonstrate the programs are of engineered microvessels made from human blood outgrowth endothelial cells and GFP-expressing human brain pericytes [20], but apply to any source of ECs and support cells.

## 2. Methods

### 2.1. Cross section algorithm description

An image analysis program for use with cross sections of engineered tissues containing microvascular networks was developed in Matlab v. 2012b (The Mathworks) using the Image Analysis Toolbox. The images used for this analysis were obtained from 10 μm cryo sections of engineered microvessels formed as previously described [20]. The sections were immunostained using an antibody against human CD31 (Dako) and imaged using a scanning laser confocal microscope. The support cells were GFP-labeled, and DAPI was used as a nuclear stain. The images were $512 \times 512$, with each pixel representing 0.877 μm. The algorithm first thresholded the CD31+ image using a several threshold value automatically generated using the *graythresh* function. The resulting binary image was then dilated and eroded (using disks of size 1 and 2 pixels, separately) to improve connectivity of CD31+ regions. The *imadjust* function was then applied to the CD31+ image and the *graythresh* function used again to threshold the image. Holes in each binary image smaller than 20 pixels in area were filled in using *imfill* and *bwareaopen*, effectively setting the lower limit on the size of lumens to 20 pixels. Several threshold values and several dilations/erosions were used to capture the most possible

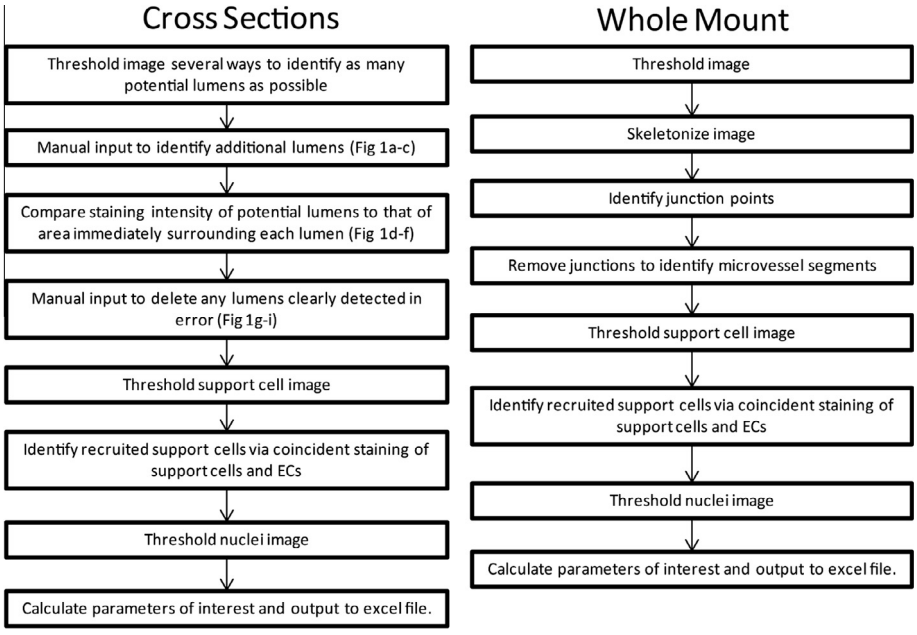


**Fig. 1.** Flow chart of processing steps for the cross-section and whole mount algorithms.

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