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Computer-aided quantification of microvascular networks: Application to alterations due to pathological angiogenesis in the hamster

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ABSTRACT

Angiogenesis is both a physiological and a pathological process of great complexity, which is difficult to measure objectively and automatically. The hamster cheek pouch (HCP) prepared for intravital-microscopy (IVM) has been used to characterize microvascular functions in many studies and was chosen to investigate microvascular characteristics observed in normal non-infected hamsters as compared to those HCPs parasitized by *Trypanosoma cruzi*. Images of HCPs captured at IVM were subjected to computer based measurements of angiogenesis and histamine-induced macromolecular (FITC-dextran) leakage with an image segmentation approach that has the capacity to discriminate between fluorescence emitted by macromolecular tracers inside the vasculature and in the extravascular space. We present such an automatic segmentation methodology using known tools from image processing field that, to our knowledge, have not been tested in IVM images. We have compared this methodology with a recently published segmentation strategy based on image intensity thresholding. Our method renders an accurate and robust segmentation of blood vessels for different microvascular scenarios, normal and pathological. Application of the proposed strategy for objective and automatic measurement of angiogenesis detection was explored in detail.

1. Introduction

Angiogenesis is a fundamental physiological process for the initiation and continuation of life with blood circulation that delivers oxygen and nutrients into the microcirculation of all tissues in the body. Angiogenesis results in macro- and microvascular networks of blood vessels that inside are completely covered by a thin layer of endothelial cells that regulate the traffic of cells (erythrocytes, leukocytes, and lymphocytes) and blood plasma proteins into the surrounding extravascular space. Angiogenesis, especially due to pathological changes as observed in tumors, may result in abnormal and complex vessel structures as well as tortuous vessels that may increase the tissue area covered by newly formed or deformed blood vessels above that of normal tissue. Therefore, quantification of angiogenesis from images is an interesting but challenging problem, whose corner-stone is vessel identification and extraction (such procedure is called segmentation). In order to perform an accurate, objective and consistent quantification of microvascular structures, automated image segmentation is mandatory. However, it appears that the use of semi-automated segmentation methodologies which are based on image intensity thresholding or manual segmentation of images dominate among studies (using commercial or open-source software) on the quantification of angiogenic induced microvascular alterations. Particularly, the impact of different segmentation strategies on vessel quantification of intravital microscopy (IVM) images of the hamster cheek pouch (HCP) has not been studied. Here, we compare the performance of the proposed methodology with that of a recently proposed one.

An image segmentation method aims at the identification and extraction of relevant objects from images. The posterior quantification and characterization of such objects strongly depend on segmentation accuracy. Furthermore, segmentation methodologies heavily rely on image modality and quality. Image processing techniques are extensively used for the analysis of microvascular structures in microscopic images, i.e. μCT and μMRI, (Gayetskyy et al., 2014; Lin et al., 2013). Tissue images can be captured from in vivo preparations, from in vitro cultures, or even from engineered tissue.

Depending on image modality and quality, several strategies for image segmentation and vessel quantification have been reported. Three segmentation approaches can be distinguished: (a) manual, (b) specialized in-house developed algorithms and (c) commercial software. For example: (i) manual characterization using ImageJ software (NIH, National Institutes of Health, Bethesda, MD) was presented in

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Ghajar et al. (2006) and Ghajar et al. (2008); (ii) several algorithms relying on image intensity thresholding were used in numerous studies (Avakian et al., 2002; Belien et al., 1999; Blatt et al., 2004; Doukas et al., 2008; Doukas et al., 2006; Niemistö et al., 2005a; Niemistö et al., 2005b; Prabhakar et al., 2015; Rodríguez, 2006; Stahl et al., 2009; Wild et al., 2000; Rabiolo et al., 2015), some were in-house developed, others used ImageJ, Photoshop (Adobe Inc., Mountain View, CA), Angiosys (TCS Cellworks, Buckingham, England), ImagePro (Media Cybernetics Inc., Silver Springs, MD), Avizo software (MCS, Bordeaux, France) among others; (iii) sometimes, when commercial software or third party services are used to process and analyze images, the corresponding methodologies are not reported (Khoo et al., 2011; Stratman et al., 2011), which limits reproducibility of results; (iv) the use of a tubeness filter (Sato et al., 1998) for μCT and μMRI segmentation was reported in Cebulla et al. (2014) and Gayetskyy et al. (2014). Moreover, reported microvascular quantification indexes include, but are not limited to number of vessels, vessel area, and vessel length, number of junctions and tortuosity of vessels.

Vessel segmentation methodologies using more sophisticated algorithms than intensity thresholding tend to produce more accurate results as reviewed by Lesage et al. (2009). Particularly, processing algorithms that highlight vessel structures in images have been presented in the past (Frangi et al., 1998; Sato et al., 1998) with remarkable results and broad adoption in the image processing community (Lesage et al., 2009). Although recent publications (Cebulla et al., 2014; Gayetskyy et al., 2014) used a tubeness filter (Sato et al., 1998) for three dimensional μCT and μMRI, to our knowledge, vesselness filtering (Frangi et al., 1998) has not been used for the evaluation of microscopy images of the HCP microvasculature. In turn, the vesselness filter (Frangi et al., 1998) has been used to process in vivo volumetric images of subcutaneous microvasculature by photoacoustic microscopy (Zhang et al., 2006).

A fully-automated threshold-based segmentation methodology for the analysis of whole mount microscopy produced images was presented by Morin et al. (2015), hereafter called the K-method. Currently, segmentation methodologies based on image intensity thresholding are the preferred choice in the processing of microvascular images. Furthermore, objective and consistent quantification of microvascular structures requires automated image segmentation strategies. In this work, we focus on automatic segmentation as a tool for the identification and quantification of microvascular vessels, specifically those observed in hamster cheek pouch (HCP) images. We propose an automatic image processing methodology and perform a head-to-head comparison with the technique proposed in Morin et al. (2015). We have chosen the method described by Morin et al. (2015) because it was suggested to be a fully-automated solution for segmentation of whole mount microscopies and it is simple to implement and available on request to the authors (Morin et al., 2015). The proposed image processing methodology is based on a vesselness filter (Frangi et al., 1998), which, in contrast to thresholding techniques, renders a more accurate and robust segmentation of blood vessels for different microvascular scenarios. Particularly, we have studied in vivo fluorescence microscopy of whole mount imaging of HCP preparations with and without angiogenesis, in order to demonstrate the capability of the proposed methodology to measure the degree of angiogenesis.

2. Materials and methods

2.1. Image acquisition

Hamsters were maintained and anesthetized according to regulations given by the local ethical committee (IBCCF, protocol-014, 23/02/2008, 021/16, 08/04/2016). Altogether, images recorded in 70 hamsters (Anilab, São Paulo, Brazil) were used. The hamster cheek pouch (HCP) was prepared and used for intravital microscopy (IVM) as described in Svensjö et al. (2009) and Svensjö et al. (2012). Briefly, the microcirculation of the HCP was observed using an Axioskop 40 microscope, objective 4× and oculars 10 × (Carl Zeiss, Germany) equipped with appropriate filters (490/520 nm, and 540/580 nm) for observations of fluorescence in epiluminescence emitted by FITC- or TRITC-dextran 150 kDa (TdB Consultancy, Uppsala, Sweden) after i.v. injection (100 mg/kg b.w.). A digital camera, AxioCam HRc, and a computer with the AxioVision 4.4 Software program (Carl Zeiss, Germany) were used for image acquisition and computation of the RFU index (relative fluorescence units) of each recorded image, see Section 2.3. Captured images correspond to a representative rectangular area (5 mm^2) of the prepared HCP. Fluorescence was recorded from time of injection of macromolecular tracer (FITC- or TRITC-dextran 150 kDa) at 5 min intervals for 30 min as described in Svensjö et al. (2009) and Svensjö et al. (2012). In the first part of our study (Part 1) the images of microvasculature obtained in a single HCP experiment were used to evaluate the influence of extravascular fluorescence on different microvasculature quantification methods after the application of 5 different doses of histamine that induced plasma leakage. In the second part (Part 2) IVM- images obtained in HCPs and captured during the first 30 min after macromolecular tracer injection were analyzed with the same quantification techniques to assess differences between untreated control HCPs ($n = 39$) and hamsters infected with genetically modified *T. cruzi* (Dm28c strain) expressing green fluorescence protein (Henriques et al., 2014), kindly provided by Dr. Cristina Henriques, Federal University of Mato Grosso do Sul. The cheek pouch tissues of anesthetized hamsters were infected with 100 μl of a suspension of 10^6 tissue culture trypomastigotes (TCT-group, $n = 23$) or inoculated with 10^6 non-infectious epimastigotes (EPI-group, $n = 6$). Seven days later the HCP was prepared for IVM as routinely done (Svensjö et al., 2009; Svensjö et al., 2012). Presence of GFP-labeled parasites or patches of GFP-deposits were exclusively observed in the HCPs inoculated with infectious forms (TCT-group). Independent studies have demonstrated that *T. cruzi* infection induces neovascularization (Guedes-da-Silva et al., 2015). The EPI-group was used as internal control, because these non-infective forms have weakly inflammatory capacity (Schmitz et al., 2009).

2.2. Image processing

Raw image data contains red-green-blue (RGB) values of fluorescence in a resolution of 1388 × 1040 pixels. Depending on contrast agent, TRITC- or FITC-dextran, the red/green color channels have predominance over the other two, see Fig. 1. All fluorescence-based indexes, see Section 2.3, are computed using only the predominant color channel.

The image processing methodology proposed in the present study, hereafter PN-method, relies on the multiscale vessel enhancement filter proposed in Frangi et al. (1998). Vesselness filters are widely used for the extraction of vessel geometry from medical images obtained by angiography, magnetic resonance and computed tomography, see references Frangi et al. (1999), Sofka and Stewart (2006), Hernandez and Frangi (2007), Lesage et al. (2009) and Zhou and Kumar (2011). In contrast to intensity thresholding, of the vesselness filter intrinsically deals with the variation in pixel intensities through space scales. This approach has so far not been used to address the segmentation of microvascular vessels.

The complete image processing strategy proposed here is automatic and consists of five steps: (i) adjust image intensities to increase image contrast, which is done by mapping the intensity values such that 1% of data is saturated at low and high intensities; (ii) apply vesselness filter, using the same set of parameters for all images (*space scale range* = [0.4, 10], *scale ratio* = 0.2, and *correction constants* $\beta = 0.5$, $c = 15$), see reference Frangi et al. (1998); (iii) create a Boolean mask, taking as one all values greater than maxP/400, where maxP is the maximum value resulting from the vessel enhancement filter; (iv) remove isolated objects from the mask with <200 in pixels size; (v) perform morphological operations of dilation and erosion, with disks structural objects of radius 2 and 3 pixels, respectively. Filter parameters were defined in order to obtain a satisfactory compromise between vessel detection and fluorescent