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Label-free optical imaging of lymphatic vessels within tissue beds *in vivo*

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Abstract

Lymphatic vessels are a part of circulatory system in vertebrates that maintain tissue fluid homeostasis and drain excess fluid and large cells that cannot easily find their way back into venous system. Due to the lack of non-invasive monitoring tools, lymphatic vessels are known as forgotten circulation. However, lymphatic system plays an important role in diseases such as cancer and inflammatory conditions. In this paper, we start to briefly review the current existing methods for imaging lymphatic vessels, mostly involving dye/targeting cell injection. We then show the capability of optical coherence tomography (OCT) for label-free non-invasive *in vivo* imaging of lymph vessels and nodes. One of the advantages of using OCT over other imaging modalities is its ability to assess label-free blood flow perfusion that can be simultaneously observed along with lymphatic vessels for imaging the microcirculatory system within tissue beds. Imaging the microcirculatory system including blood and lymphatic vessels can be utilized for imaging and better understanding pathologic mechanisms and treatment technique development in some critical diseases such as inflammation, malignant cancer angiogenesis and metastasis.

Index Terms

Lymph vessel; optical coherence tomography; Lymph; label-free imaging; Multi-scale Hessian filters

I. Introduction

The vascular system in vertebrates consists of cardiovascular and lymphatic sub-systems, both of which are highly branched tree-like tubular structures. The main role of cardiovascular system is to deliver oxygen, nutrition, fluid and signaling molecules required by living tissue via arteries and arterioles, for which the exchange usually happens at capillary beds. Venules and veins then collect deoxygenated blood from the tissues back to the heart. Due to blood pressure and osmotic gradients, some part of plasma leaks from the

capillaries into the interstitial space. The protein-rich interstitial fluid (lymph) is collected back by lymphatic capillaries to maintain tissue fluid homeostasis.

The lymphatic system consists of unidirectional, thin-walled capillaries and larger vessel network that drains lymph fluid from extracellular space within organs into larger collecting ducts. Lymph fluid moves from lymphatic capillary bed into a larger vessels covered by smooth muscle cells, called precollector vessels. Then, precollector vessels fuse with collecting lymphatic vessels, which have a basement membrane, continuous interendothelial junctions and bileaflet valves, in addition to a layer of smooth muscle cell. Contraction of smooth muscle cell layer and surrounding skeletal muscles as well as arterial pulsations are necessary for lymph movement, while valves prevent lymph backflow [1]. Finally, lymph is returned to venous circulation through thoracic duct. The lymphatic system also includes lymphoid organs such as sentinel lymph nodes (SNL), tonsils, Payer's patches, spleen and thymus. The lymph nodes (LNs) are glands that collect lymph from vessels and their role is to entrap potentially harmful substances such as bacteria and cancer cells in the body. SLNs are the primary nodes that drain waste from developing tumors.

The lymphatic system usually develops in parallel to the blood vessels in the skin and in most internal organs and is not present in central nervous system, bone marrow and avascular structures, such as cartilage, epidermis and cornea. Besides draining lymph fluid from extracellular spaces, other roles of lymphatic system include absorbing lipids from intestinal tract, maintaining fluid hemostasis and transporting antigen-presenting cells and leukocytes to lymphoid organs. Also, lymphatic system plays an important role in the development of several diseases such as cancer, lymphedema, some inflammatory conditions and allergies [2–4].

A. Imaging and visualization of lymphatic vessels

Due to the lack of detection and monitoring methods, lymphatic vessels are called “the forgotten circulation” and their role was mostly ignored [5]. Although lymphatic vessels were first described in the 17th century, the first reported detection method was not until mid-20th century and the first lymph-specific molecular marker was introduced only a decade ago. The field of imaging and visualization of lymphatic vessels can be divided into three main categories: lymph-drainage tracing, active cell targeting biomarkers and label-free. In lymph-drainage tracing methods, a contrast agent is required for injection into the tissue, and its uptake pathway by lymphatic vessels and SLNs are then become apparent. Active cell targeting methods involve identification and development of proteins and antibodies that bind to lymphatic-specific cells. Label-free methods involve no tracers or markers and use assumptions about certain characteristics of lymph fluid and their appearance/vessel lumen to image the lymphatic vessels.

1) Lymph drainage tracing methods—Since one of the roles of lymphatic system is to collect toxic and waste material from interstitial fluid, most of the developed clinical lymphatic imaging techniques was based on injection of contrast agents or dyes in the tissue or direct injection into the lymphatic vessels, which can provide contrast for uptake path by lymph vessels and nodes. Kimmonth pioneered direct visualization of lymphatic vessels

(lymphangiography) by injecting radiopaque materials into small lymphatic vessels. He then used X-ray imaging to visualize the uptake path [6]. Later on, several contrast-enhanced and labeled lymphangiography agents were developed for standard imaging modalities such as X-ray, computed tomography (CT), magnetic resonance imaging (MRI), ultrasound and optical imaging. In contrast-enhanced lymphangiography, an iodinated contrast agent such as blue dye [7], Iotasul (Sshering AG, Berlin, Germany) [8] or Iopamidol (Nihon Schering, Osaka, Japan) [9] is either injected directly into a lymph vessel or indirectly into intradermal area for X-ray or computed tomography (CT). Although CT scans differentiated iodine concentration and LN morphology in normal and malignant LN, their application was limited due to the fast drainage of contrast agents from blood vessels that limited the imaging time in clinic. More recent development of bismuth sulfide nanoparticle CT agents with longer circulating half-life addressed some of the problems while their long-term toxicity is a major concern [10].

Positron emission tomography (PET) with beta-emitting isotopes (^{18}F -fluro-2-deoxy-D-glucose, FDG) provided higher sensitivity specifically when combined with CT for better spatial resolution [11]. Lymphoscintigraphy with subcutaneous or intradermal injection of $^{99\text{m}}$ -technetium (TC- $^{99\text{m}}$) is currently the “gold standard” for lymphatic imaging in clinic and the most common nuclear method of imaging the lymphatics that enables visualization of the lymphatic network using single-photon emission computer tomography (SPECT) [12–14]. The injected compounds are taken up by lymphatic vessels and transported to draining LNs where they are phagocytosed by nodal macrophages. In lymphoscintigraphy, serial images are acquired using a gamma scintigraphic-camera and quantified by analyzing the clearance rate from the injection site [15]. However, this technique requires rejection of scattered gamma photons and a large number of photons remain undetected and the scanning times can be long [16]. Another major disadvantage of this technique is poor temporal and spatial resolution, which limits direct visualization of detailed lymphatic anatomy and function [17].

MRI, unlike nuclear imaging techniques, does not require ionizing radiation exposure. MR lymphangiography is performed by intravenous or interstitial injection of gadolinium-labeled diethylene-triaminepentaacetic acid (Gd-DTPA), Gd dendrimers or liposomes and iron oxide particles [18–20]. However, besides high costs and low sensitivity of MRI for lymphangiography, MR lymphatic specific contrast agents are not clinically approved and some concerns regarding safety issues remain. Also, most CT and MRI lymphatic imaging is limited to enlarged LNs and vessels and normal LNs are below system sensitivity, despite the large lymph flow [20].

Ultrasound has been used to investigate lymphadenopathy, enlargement and LNs [21]. Also, color Doppler ultrasound (CDUS) provided functional imaging of the LNs which can be used to classify nodes as being reactive, metastatic, tuberculous, cystic or enlarged secondary to lymphoma [22]. Intravenous injection of ultrasound micro-bubbles allowed the measurement of flow within a LN [23]. Using this technique, benign and malignant lymphadenopathy was distinguished such that the degree and duration of contrast enhancement as well as the number of vessels recorded was higher within the malignant

nodes [24]. The main disadvantages of ultrasound are its operator dependent, poor spatial resolution and its limited use in the thorax and deep retroperitoneum [20].

Photoacoustic tomography (PAT) is an image modality that is based on thermal expansion induced by pulsed laser and subsequent detection using ultrasound transducers. Based on unique absorption coefficients of oxygenated and deoxygenated hemoglobin, PAT allows high resolution imaging red blood cells. However, lymph is optically transparent and cannot be imaged using label-free PAT, therefore dye injection is required. Since Evan's blue has an absorption peak at ~610-nm, its injection into the tissue provides contrast for PAT using a 640-nm laser source. Therefore, PAT using two lasers at 523-nm and 610-nm for blood vasculature and lymphatic vessels, respectively, along with injection of Evan's blue allowed simultaneous imaging blood and lymphatic vessels in mouse cologne [25]. Other nanoparticle-based PAT contrast agents such as carbon nanotubes [26], gold nanobeacons [27] and gold nanocages [28] allowed imaging SLNs about 2–3 cm deep in rats and mice.

Optical imaging of lymphatic vessel drainage is gaining increased interests because it does not involve any radiation and can achieve very high resolution [29]. Indocyanine green (ICG) is an unconjugated free emissive fluorophore which has a significant Stoke's shift that allows fluorescence measurement at 830 nm if excited at 780 nm. ICG is one of the popular dyes for non-invasive near-infrared (NIR) detections of tumors in both research and clinic (approved in humans for assessing cardiac and hepatic function as well as lymphatic flow and SLN imaging [30]). Alexa 705, IRDye780, Cy7 and Cy5.5 are among other NIR organic fluorophores used for detecting lymphatic drainage which can also be conjugated with bioactive molecules to effectively enter and remain in the lymphatic system [31, 32]. Although most of the organic NIR fluorophores suffer from weak fluorescent signal through deep tissue sections, research in designing high-emission dipole strength fluorophores still continues. Quantum dots (Qdots) are the result of the recent advances in the field of nanotechnology in fabricating nano-sized semiconductor crystals [33]. The size of Qdots can be fine-tuned ranging between 5–20 nm to control the emission wavelengths. The main advantage of Qdots over organic fluorophores is their strong brightness which allows for deeper penetration in the tissue [34]. Using NIR Qdots, *in vivo* lymphatic flow draining and SLNs have been successfully demonstrated in superficial tissues [35–37].

2) Active targeting and molecular imaging using targeting biomarkers—

Immunohistochemistry (IHC) is the process of detecting antigens (e. g. proteins) in cells within a tissue section using specific antibodies. IHC, also known as a bridge between immunology, histology and chemistry, is a valuable tool in the diagnosis and research of infectious and neoplastic diseases. The antigen-antibody (Ag-Ab) is demonstrated with a colored histochemical reaction visible by light microscopy or fluorochromes with ultraviolet light [38]. Since lymphatic endothelial cells are different from blood vascular endothelial cells, lymphatic vascular-specific molecules have been discovered and developed for identification of lymphatic vessels in tissues. These molecules include vascular endothelial growth factor receptor-3 (VEGFR-3) [39], Prospero-related homeodomain transcription factor Prox1 [40], the membrane glycoprotein podoplanin [41], and lymphatic vessel hyaluronan receptor-1 (LYVE-1) [42]. The receptor tyrosine kinase (RTK) VEGFR-3, one of the first discovered lymphatic endothelial markers [2], is activated by VEGF-C and

VEGF-D of the VEGF family of growth factors [43]. LYVE-1 is one of the most specific and widely used lymphatic endothelial markers that are expressed in a subset of endothelial cells in the large central veins [44]. In adults, LYVE-1 expression decreases in collecting lymphatic vessels and remains high only in lymphatic capillaries [45].

Protein-binding detection techniques are not limited to *ex vivo* samples. *In vivo* imaging of lymphatic vessels in development, wound healing, inflammation and tumor metastasis were made possible by expression of enhanced green fluorescent protein- (EGFP-) luciferase fusion protein under the endogenous transcriptional control of VEGFR3 gene. This technique allowed whole-body imaging and monitoring of physiological and pathological lymphangiogenesis *in vivo* [46]. The active targeting strategies can also be applied to other imaging modalities such as ultrasound and PET. In ultrasound active targeting contrast imaging, antibodies, peptides or other molecules are attached to micro-bubble shell. Some examples include lipid shell microbubbles attached anti-OCAM-1 monoclonal antibody for imaging acute cardiac allograft transplant rejection in rats, microbubbles targeted to vascular endothelial growth factor receptor type 2 (VEGFR2) for imaging tumor angiogenesis in murine tumor models and microbubbles with RGD peptide for thrombosis and angiogenesis targeting [47–49]. Also, feasibility of using recombinant human adenoviral vectors to detect nodal metastases of prostate cancer was demonstrated with adenovirus-mediated gene expression PET imaging [50].

3) Label-free imaging of lymphatic system—Although most of the existing lymphatic imaging techniques require contrast agents, their toxicity and side effects can limit their applications. To the best of our knowledge, the only label-free methods for imaging lymphatic vessels *in vivo* are optical coherence tomography (OCT) and laser speckle imaging (LSI). However, LSI is not depth-resolved and the resolution is not as high as OCT [51]. OCT has been widely used to non-invasively provide high-resolution, depth-resolved cross-sectional and three-dimensional (3-D) images of highly scattering samples [52–54]. Functional and hemodynamic information in OCT can be acquired in addition to the structure. Optical micro-angiography (OMAG) is a label-free non-invasive imaging and processing method to obtain 3D blood perfusion map in microcirculatory tissue beds *in vivo* using FD-OCT [55, 56]. Ultrahigh-sensitive OMAG (UHS-OMAG) is an variation of OMAG technique, capable of imaging microvasculature down to capillary level, in which data acquisition is based on repeated B-scan (frame) acquisition at the same spatial location [57, 58], and then separating static scatters (e.g. structure tissue) and dynamic scatters (e.g. moving red blood cells within patent vessels) by the use of OMAG algorithms, such as in [59]. By synergistically utilizing both the amplitude and phase information of complex OCT signals, the OMAG technique is sensitive to both the axial and transverse blood flows, providing blood flow map at small vessels and capillaries as well as larger vessels. UHS-OMAG has been applied to visualize blood perfusion and micro-vasculature map in various living tissue samples such as retina [60], cerebral [61], renal microcirculation [62] and skin [63]. Hemodynamic quantification can be extremely useful in cancer, stroke and some other disease which involve vasculature.

Although contrast-based imaging LNs has been demonstrated using gold nanoparticles uptake [64], label-free imaging of intact SLNs [65] and lymph vessels [66] have been

demonstrated *in vivo*. Since the lymph fluid is clear and transparent, lymphatic vessels appear as reduced scattering (system noise level) vessel-like areas in OCT structure cross-section images. The origin of these reduced-scattering connected tubular structures in the skin has been already confirmed by intra-dermal injection of Evan's blue dye and monitoring the uptake pathway by surrounding lymph vessels into the SLN. Therefore, these lymph vessels can be visualized by applying a lower threshold on the OCT intensity image [66, 67]. However, the intensity-threshold technique does not take the physical shape of the vessels into account, therefore not robust to intensity variations and noise caused by speckle and light absorption in the structure. These limitations increase the chances of segmentation error and false alarms in identifying lymphatic vessels. We have developed an automatic method for segmenting lymphatic vessel in OCT structure images using filtering technique and vesselness models based on Hessian multi-scale filters [68, 69]. Hessian filters estimate tubular and vessel-like structures and the multi-scale approach allowed segmenting vessels with different size. In this paper, we demonstrate the capability of OCT for non-invasive label-free imaging of lymphatic vessels along with blood flow perfusion within tissue beds and a segmentation method to extract lymphatic vessel lumen. Simultaneous imaging and visualization of blood and lymphatic vessels will be useful for better understanding and observing tissue structures and microcirculatory response to some pathological cases such as wound healing, infection and cancer angiogenesis.

II. Materials and Methods

A. System setup

In the OMAG/OCT system, we utilized a superluminescent diode (SLD) with the center wavelength of 1310 nm and bandwidth of 65 nm, delivering an axial resolution of $\sim 12 \mu\text{m}$ in air. The schematic of the imaging system is shown in Fig. 1A, based on spectral domain OCT configuration. An optical circulator was used to couple the light from the SLD into fiber-based Michelson interferometer. In the reference arm of the interferometer, the light was focused onto a stationary mirror. In the sample arm of the interferometer, the light was focused by a microscopy objective lens with 18-mm focal length, providing $\sim 5.8 \mu\text{m}$ lateral resolution. Backscattered light from the sample and the reflected light from reference mirror were recombined by the 2×2 optical fiber coupler, and then routed to a home-built high-speed spectrometer via optical circulator. In the design of the spectrometer, a collimator with the focal length of 30 mm and a 14-b, 1024-pixels InGaAs line-scan camera (SUI, Goodrich Corp) were used. The camera speed was 47 000 lines per second and the measured SNR was ~ 105 dB with a light power on the sample at ~ 3 mW. The spectral resolution of the designed spectrometer was ~ 0.141 nm that provided a detectable depth range of ~ 3.0 mm on each side of zero-delay line. Since the wavelength of the light source is invisible to the human eye, a 633-nm laser diode was used as a guiding beam to locate the imaging position. This reference helps adjust the sample under the OMAG/OCT system and image the desired location.

The scanning protocol was based on three-dimensional ultrahigh-sensitive optical microangiography (UHS-OMAG) technique [57]. The x-scanner (fast B-scan) was driven with a saw tooth waveform and the y-scanner (slow C-scan) was driven with a step function

waveform. The fast and slow scanners covered a rectangular area of $\sim 2.2 \times 2.2 \text{ mm}^2$ on the sample. Each B-scan consisted of 400 A-lines covering a range of $\sim 2.2 \text{ mm}$ on the sample. The duty cycle of the saw tooth waveform rising edge was set at $\sim 80\%$ per cycle, which provided a B-scan frame rate of ~ 94 frames per second. The C-scan consisted of 400 scan locations with B-scan repetition of 8 frames per location for flow imaging and quantification. The total size of the data set was 1.28×10^6 A-lines. In order to cover a large field of view, multiple 3-D scan were acquired and the sample was translated using a mechanical stage. This allowed imaging an area of $\sim 1.5 \times 1.5 \text{ cm}^2$ on the mouse ear pinna.

B. Segmentation of lymphatic vessels from OCT structure images using Hessian vesselness filtering

The segmentation algorithm is based on multi-scale Hessian filters as previously described [69] and here we briefly overview the method. In our method, the local behavior of the 2nd order gradient image (Hessian matrix) is used to identify the boundaries of tubular low-scattering lymphatic vessels and contrasting them from background and other scattering tissue. The multi-scale nature of the method allows identifying vessels of different size. Based on linear space theory, the 2nd order derivative of a discrete matrix (digital image) can be estimated by convolving (filtering) the original image with the 2nd derivative of a Gaussian kernel. The advantage of this substitution is that the derivative operation reduces to linear filtering and simplifies the implementations on hardware and real-time systems.

The second order derivative can be expressed as

$$\delta x^T H(I)_s \delta x = \left(\frac{\partial}{\partial x} \right) \left(\frac{\partial}{\partial x} \right) I(x, s) = s^{2\gamma} I(x, s) * \frac{\partial^2}{\partial x^2} G(x, s) \quad (1)$$

where $I(x, s)$ is the original image at scale s and location x , $H(\cdot)_s$ is the gradient vector of the

image at scale s , γ is the derivative normalization parameter and $G(x, s) = \frac{1}{\sqrt{2\pi}s^2} e^{-\frac{\|x\|^2}{2s^2}}$. By analyzing the eigenvalues and eigenvectors of the Hessian matrix, the principal direction of the local structure can be extracted which is the direction of the smallest curvature (along the vessel). By definition, the eigenvalues and vectors of the Hessian matrix are given by solving the following equations

$$H_s e_{s,i} = \lambda_{s,i} e_{s,i} \quad (2)$$

where $\lambda_{s,i}$ is the i -th eigenvalue at scale s corresponding to normalized eigenvector $e_{s,i}$ and $(|\lambda_3| \quad |\lambda_2| \quad |\lambda_1|)$ for a three-dimensional structure. For an ideal tubular structure $|\lambda_3| \approx 0$, $|\lambda_3| \ll |\lambda_2|$ and $\lambda_2 \approx \lambda_1$. Based on the second order ellipsoid, two geometric ratios are defined

$$R_B = \frac{|\lambda_1|}{\sqrt{\lambda_2 \lambda_3}} \quad (3)$$

and

$$R_A = \frac{|\lambda_2|}{|\lambda_3|} \quad (4)$$

The first ratio (R_B) accounts for the deviation from a blob-like structure but cannot distinguish between a line-like and a plate-like pattern. The second ratio refers to the largest cross-section area of the ellipsoid that can distinguish between plate-like and line-like structures since it is zero in the latter case. In order to distinguish background noise where no structure is present, Frobenius matrix norm is utilized. This measure is derived from eigenvalues of Hessian matrix by

$$R_C = \|H\|_F = \sqrt{\sum_{j \leq D} \lambda_j^2} \quad (5)$$

where D is the dimension of the image. This measure will be low in the background where no structure is present and the eigenvalues are small for the lack of contrast. In regions with high contrast compared to the background, the norm will become larger since at least one of the eigenvalues will be large.

The vesselness function at scale s is defined as

$$v_0(s) = \begin{cases} 0 & \text{if } \lambda_2 > 0 \text{ or } \lambda_3 > 0 \\ \left(1 - e^{-\frac{R_A^2}{2\alpha^2}}\right) * e^{-\frac{R_B^2}{2\beta^2}} * \left(1 - e^{-\frac{R_C^2}{2\theta^2}}\right) & \text{otherwise} \end{cases} \quad (6)$$

where α , β and θ are thresholds which control the sensitivity of the line filter to the measures R_A , R_B and R_C . The idea behind this expression is to map the features into probability-like estimates of vesselness according to different criteria.

The vesselness measure is analyzed at different scales. The response of the line filter will be maximal at a scale that approximately matches the vessel size.

$$V_0(\gamma) = \arg \max_s v_0(s, \gamma), s_{min} < s < s_{max} \quad (7)$$

where s_{min} and s_{max} are lower and upper bound in the range of scale (vessel sizes).

By utilizing matrix properties of eigenvalues, the computational complexity of this approach is reduced to simple 2-D filtering with already-known Gaussian derivative kernels at multiple scales. Since all of the operations are performed on matrices, the computation can be parallelized on the processing unit and real-time segmentation can be performed on the structure images. More detail on parallel implementation of the proposed method is given in [69].

III. Experimental results and discussions

A. Monitoring simultaneous response of blood and lymphatic vessels in wound healing

Non-invasive *in vivo* images were acquired from the pinna of a healthy ~6 weeks old male hairless mouse (CrI:SKH1-Hr^{hr}) weighting approximately 25g. During the experiment, the

mouse was anesthetized using 2% isoflurane (0.2 L/min O₂, 0.8 L/min air). The ear was kept flat on a microscope glass using a removable double-sided tape. The experimental protocol was in compliance with the Federal guidelines for care and handling of small rodents and approved by the Institution Animal Care and Use Committee (IACUC) of the University of Washington, Seattle. A photograph of a mouse ear pinna captured by a digital camera is shown in Fig. 1B where the rectangle box shows a typical field of view and scanning range for OMAG/OCT system, which is 2.2×2.2 mm² in the current setup. In order to scan a larger field on the ear, we used a mechanical translating stage to move the tissue sample to the desired location and after acquisition and processing, the mosaics were stitched together to form a larger image.

The ear pinna is consisted of two layers of skin and a layer of cartilage in the middle which holds the skins together. We used this model because it was a convenient *in vivo* model given the penetration depth limitations of optical imaging; we were able to thoroughly image the structures within tissue, including blood and lymphatic vessels. Using this model allowed studying and monitoring some phenomena such as vasculature remodeling and response to injuries. Under normal conditions, majority of the lymphatic vessels and capillaries are very small and fall below the sensitivity of our system. However, immediately after we created a biopsy punch, we observed that the size of lymphatic vessels enlarged. Perhaps this enlargement was due to the inflammatory and immune response at the injury site. The biopsy punch model was very similar to the one previously reported by Jung et al. [72] where they created a biopsy punch and complete removal of tissue on the ear skin flap and monitored the vascular remodeling and healing process along the time. In this paper, we also monitor the response of lymphatic vessels along with blood vessels to that injury model. Using our OCT system, we studied simultaneous response of blood and lymphatic vessels to the biopsy punch injury. 3-D images were acquired at each location covering 2×2 mm² and a large field of view was covered by mechanically shifting the sample under the system. The 3-D images at each location were processed separately and the lymphatic vessels were segmented using Hessian vesselness filters. Fig. 2A–C show blood vessels, lymphatic vessels and structure cross-section images of a selected location on the ear, respectively. Fig. 2D–F show the volumetric blood vessels and lymphatic vessels map, respectively, and the cross-section shown in Figure 3C is also located. The lymphatic vessels are coded by green color and blood flow perfusion is coded with orange. By combining blood flow perfusion and lymphatic vessels from Fig. 2D and 2E, Fig. 2F was generated that shows three-dimensional complications of circulatory system. For better visualization and quantification, maximum intensity projection (MIP) maps of the vasculature were created. Figs. 2G–H show the MIP of blood flow perfusion, lymphatic vessels and their combinations. For Fig. 2H, lymphatic MIP was color-coded based on the vessel diameter. The vessel diameter was estimated using distance transform.

Figs. 3A–D show the process of vasculature remodeling and lymphatic vessel response to the wound. Each image is combination of 9 OCT-OMAG mosaics acquired around the wound area. Blood vessels are gray-coded and lymphatic vessels color-coded with diameter-dependent orange color map. The peak activity of lymphatic vessel enlargement was observed around ~1 week after inducing the wound. This observation is in agreement with

the wound healing phases during the inflammatory phase [46, 73, 74]. The immediate response of lymphatic vessels after inducing the punch (Fig. 4A) was mainly concentrated around the wound, collaterals and downstream of the injured vessels. However, the size of the lymphatic vessels had significantly increased after one week (Fig. 3B) and it was not only around the wound, but also at farther locations on the ear. Then, the size and distribution of lymphatic vessels reduced on Day15 and Day22 (Figures 3C and D, respectively) and was mainly around the wound area, maybe due to inflammation. Wound healing and tissue regeneration is associated with angiogenesis. Angiogenesis can be observed starting on Day8, where new capillary network is formed around the wound area in the form of granulation tissue and eventually mature in the form of new vessels inside the wound.

B. Lymphatic vessel quantification

In order to quantify the lymphatic vessel response to biopsy punch, two parameters were measured.

- *Lymphatic vessel area density (LVAD)* was defined as the area of the segmented lymphatic vessels in the projection view divided by the area of the ear given by

$$LVAD = \frac{\text{Total area of the lymphatic vessels}}{\text{Total area of the ear}} \quad (8)$$

- *Lymphatic vessel diameter (LVD)* was quantified using Euclidean distance transform (EDT) of the binarized lymphatic vessel en-face projection map.

The EDT of each pixel in a binary image measures the Euclidean distance between that pixel and the nearest nonzero pixel element of that image. First, the lymphatic vessel map was binarized above a threshold. Then, the vessel center-line was extracted by skeletonizing the binary vessel map. Finally, the distance transform of the intensity-inverted lymphatic vessel map was estimated at each vessel centerline pixel. EDT at the vessel centerline is an estimate of the vessel diameter. For each imaging time-point after inducing the biopsy punch, LVAD, mean value and standard deviation of LVD were calculated. The lymphatic vessel response was observed immediately after creating the wound. Peak LVAD and LVD were observed on Day8 and eventually at Day22 they decreased below values of Day1. The quantification values are plotted in Figures 3 E, F for LVAD and LVD, respectively.

C. Imaging sentinel lymph node

Current gold standard for assessing the stage of breast cancer is SLN biopsy [75]. The biopsy requires complete removal of SLN with the help of an invasive dye injection. However, it may not be necessary to completely remove SLN if they could be non-invasively imaged [76]. Removal of SLNs may lead to post-operative complications such as lymphedema and sensory nerve injury [77]. Since light penetration limits OCT imaging depth to ~1.5mm, imaging intact LNs required surgical exposure of the node. Although OCT probes can be mounted in a medical needle [78] for deeper tissue imaging, exact location and desired microstructures and blood flow perfusion can be better assessed by surgical exposure. A three-month-old C57 BL/6 mice (~25 g) was immobilized in a

stereotaxic stage and was anesthetized with vaporized isoflurane (0.2L/min oxygen and 0.8 L/min air) while the body temperature was kept constant at normothermia using a heating pad. The auxiliary lymph node was surgically exposed by pulling aside the skin and surrounding tissue. Then the sample was placed under the probe beam and we performed OCT/OMAG imaging on the exposed SLN *in vivo*. Figure 4A shows a structure cross-section where lymph vessels appear as transparent vessel-like structures. Figure 4B shows the blood flow perfusion cross-section processed using OMAG algorithm. The vessel lumen segmentation is shown in Figure 4C. The volume rendering of blood flow perfusion (color-coded in orange) and lymphatic vessels (color-coded with green) are shown separately in Figures 4D and 4E and overlaid onto Figure 4F. Figure 4G shows an X-Z cross-section view overlaying blood and lymphatic vessels on the structure image that the boundaries of the lymphoid lobule are visible (red marker). These vasculatures show an artery/vein pair and a lymphatic vessel around the node and branch of these vessels enter the node.

IV. Conclusions

In this paper, we reviewed the capability of using OCT for label-free imaging blood flow perfusion and lymphatic vessels and developed an automatic filtering technique and vesselness model based on Hessian multi-scale vesselness filters for segmenting lymphatic vessel in OCT structure images. Hessian filters estimate tubular and vessel-like structures and the multi-scale approach allowed segmenting vessels with different size. Using this segmentation technique along with optical micro-angiography (OMAG) allows for label-free non-invasive simultaneous visualization of blood and lymphatic vessels *in vivo*.

In order to observe the capability of our system, we created a biopsy punch wound on the mouse ear and monitored the dynamics and healing process of lymphatic and blood vessels around the wound. Although no significant lymph was detected on the baseline image, immediately after inducing the punch the lymphatic vessels were significantly enlarged around the wound and in the downstream tissue. In order to quantify the lymphatic vessel response to biopsy punch, two parameters were measured: lymphatic vessel area density and lymphatic vessel diameter. The lymphatic vessel response was observed immediately after creating the wound. Peak LVAD and LVD were observed on Day8 and eventually at Day22 they decreased below values of Day1.

Lymphatic system plays an important role in cancer, immune system response, inflammatory disease, wound healing and tissue regeneration. Development of imaging techniques and visualization tools for lymphatic system is valuable in understanding the mechanisms and studying therapeutic methods in related disease and tissue response. Due to lack of label-free non-invasive imaging techniques, lymphatic vessels have been ignored and their behavior in the disease and their response to treatment methods is not well known. Label-free non-invasive imaging and segmentation of SLNs and lymphatic vessels can be used in different applications such as dermatology, cosmetic and beauty, cancer, wound healing and infectious disease where lymphatic system plays an important role in delivering immune response and draining waste. The observed lymphatic vessels on the OCT structure images are directly related to the resolution of the system. By increasing the system

resolution, smaller lymphatic vessels and capillaries can also be observed and eventually segmented using our proposed method.

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Biographies

Siavash Yousefi, was born in Iran in 1983. He received his B.S. in electrical engineering from University of Tehran, Iran, in 2006, and M.S. in electrical and computer engineering from Oregon State University, Corvallis, OR, in 2008. He is currently a Ph.D. student at University of Washington, Seattle, WA. His research interests include biomedical optics, optical coherence tomography and medical science translational research.

Zhongwei Zhi received a B.S.E. in Biomedical Engineering from Huazhong University of Science and Technology in 2009. He is currently working toward the Ph.D. degree in the Department of Bioengineering, University of Washington at Seattle.

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Fig.1.

(A) Schematic diagram of the imaging system, where PC represents the polarization controller. (B) Typical mouse ear pinna flattened on the imaging platform. The biopsy punch size is 1mm. The rectangular box shows typical scanning size of about $2.2 \times 2.2 \text{ mm}^2$.

Fig. 2.

Cross-section images of processed blood vessels (A), segmented lymphatic vessels (B) and OCT structure B-mode (C) and their corresponding 3-D volume rendering of blood vessels (D) and lymphatic vessels (E). The cross-section location is also marked. (F) Combined blood vessels (orange color) and lymphatic vessels (green color) in volume rendering. (G) Maximum intensity projection map of blood vessels. (H) Maximum intensity projection map of lymphatic vessels. (I) Combined blood vessels (gray-scale) and lymphatic vessels (color-coded) maximum intensity projection map. The lymphatic vessels color map is a function of vessel diameter and maximum intensity value from (H). (Scale bar = 1mm)

Fig. 3. (A–D) Simultaneous imaging blood and lymphatic vessels in the healing process. (E) Lymphatic vessel area density and (F) lymphatic vessel diameter quantification of wound healing in the mouse ear pinna model. The labels A–D on figures (E) and (F) are corresponding to their microvasculature maps of figures (A–D).

Fig. 4. (A) Structure cross-section. (B) Blood flow perfusion. (C) Lymph vessels. (D) Overlaying blood and lymphatic vessels on a structure cross-section. (E) Volume rendering of blood vessels. (F) Volume rendering of lymphatic vessels. (G) Overlaying blood and lymphatic vessels. (H) Location of lymph node with the blood and lymph capillaries. (Scale bar = 300µm)