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**Abstract.** We introduce flow optical projection tomography, an imaging technique capable of visualizing the vasculature of living specimens in 3-D. The method detects the movement of cells in the bloodstream and creates flow maps using a motion-analysis procedure. Then, flow maps obtained from projection taken at several angles are used to reconstruct sections of the circulatory system of the specimen. We therefore demonstrate an *in vivo*, 3-D optical imaging technique that, without the use of any labeling, is able to reconstruct and visualize the vascular network of transparent and weakly scattering living specimens. © *2011 Society of Photo-Optical Instrumentation Engineers (SPIE)*. [DOI: 10.1117/1.3640808]

Three-dimensional optical imaging is increasingly being used in many areas of biology, allowing the analysis of complex and diverse models. Aside from commonly used confocal and multiphoton microscopies, other three-dimensional imaging techniques, such as single-plane illumination microscopy[1] and optical projection tomography (OPT)[2] are emerging as valuable tools for a wide range of biological applications that require the visualization of intact and entire organisms or biological tissues. OPT is a relatively simple and low-cost technique that is particularly suitable for studying millimeter-sized samples. Similarly to x-ray computed tomography, OPT is based on the acquisition of a sequence of optical transmission (or fluorescence) images of the sample at several orientations. The acquired images, or projections, are combined to reconstruct the 3-D volume of the sample, typically using a backprojection algorithm. OPT is currently evolving from a method to image small chemically cleared samples to a three-dimensional imaging technique for living specimens.[3–5]

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In this paper, we report a new contrast mechanism in OPT, given by the movement of cells present in bloodstream. Looking at a living transparent or weakly scattering sample it is possible to observe the flow of the blood cells. Therefore, by acquiring several time frames of the specimen and applying a motion-analysis algorithm, it is possible to obtain a map of the sample vasculature.[6] Here, we show that by mathematical processing the vascular maps obtained at different angles it is possible to produce and visualize a 3-D casting of the vasculature of the specimen, noninvasively and without the need for any fluorescent probe. This results in a low-cost, label-free, three-dimensional imaging technique, which we will hereafter call flow-OPT. The present study has been performed on a juvenile zebrafish (*Danio rerio*), a model organism widely used in developmental biology[7] and oncology.[8] However, these concepts could be generally extended to other transparent and weakly scattering living samples.

The flow-OPT system is depicted in Fig. 1. The light emitted by a high-power white light-emitting diode (MCWHL2, Thorlabs, Delaware) is passed through a diffuser (DG10-1500-MD, Thorlabs, Delaware) and projected on the specimen with a telecentric lens (TC2309, OptoEngineering, IT). The light transmitted through the sample is imaged by a 5X telecentric objective (NT56-986, Edmund Optics, Delaware) on a CMOS camera (B771U, Pixelink, California), which is operated at half resolution ($640 \times 480$ pixels) to acquire 100 fps. Telecentric optics are used in order to keep a constant magnification through the specimen.

The sample is then rotated with a stepper motor (8MR151, Standa, LT) around its longitudinal axis with steps of 0.9 deg over 360 deg, leading to 400 acquisitions at different angles. Typically, each frame acquisition lasts 3 ms, resulting in ~3-min measurement time. The system is mounted on a passive-vibration isolation breadboard in order to prevent movement of the optical setup during the measurement.

The sample consists of a juvenile Casper zebrafish[9] transgenic for *tg(fli1:GFP)y1*[10] (30 days postfertilization, ~1 cm long). This zebrafish does not present skin pigmentation and is optically weakly scattering. The zebrafish is anesthetized with Tricaine mesylate, mounted in a 2-mm-diam fluorinated ethylene propylene (FEP) tube (FT2X3, Adtech Polymer Engineering, UK) with 1.5% low melting point agarose and then immersed in a water cell. Undistorted images of the zebrafish are obtained taking advantage of the fact that FEP matches the refractive index of the surrounding water.[11] Furthermore, the tube enables the rotation of the sample and prevents its movement. It is particularly important to avoid movement of the sample in flow-OPT because it would cause artifacts in the reconstruction. The heartbeat could cause movement of the thoracic region of
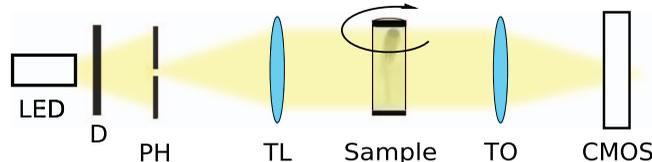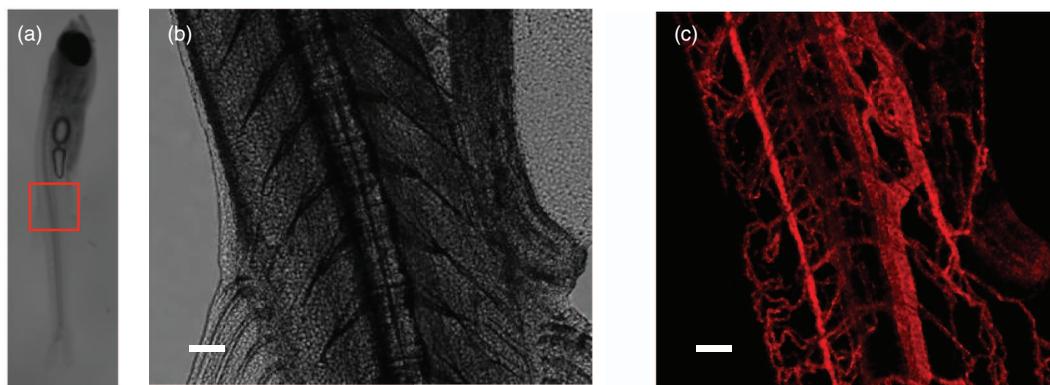


**Fig. 1** Scheme of the experimental setup for flow-OPT (D: diffuser; PH: pinhole; TL: telecentric illumination lens, TO: telecentric objective.)

**Fig. 2** (a) The acquisition region is highlighted in red on the picture of the juvenile Casper zebrafish; (b) raw image of the trunk (also shown in Video 1); (c) contrast map obtained by combining $N = 40$ raw images. Scale bars are 100 $\mu$m. (Video 1, QuickTime, 65.3 KB) [URL: http://dx.doi.org/10.1117/1.3640808.1]

the zebrafish, but we verified that the sample displacement was negligible in the acquisition area (trunk and tail) by comparing the first and last acquired images. Although the zebrafish could recover after the measurement, it was terminally anesthetized with ethyl-m-aminobenzoate metasulphonate (MS-222, Sigma, Missouri) at the end of the acquisition. After fixation with 4% paraformaldehyde, the fish trunk was cut with a vibrotome at a nominal thickness of 200 $\mu$m. The study was approved by the ethical committee of IFOM (protocol 10/003).

A picture of the sample in shown in Fig. 2(a) and an acquired raw image of the zebrafish trunk in presented in Fig. 2(b) and Video 1. Looking at successive time frames, the movement of the cells in the bloodstream can be observed (see Video 1) and the regions of blood flow can be extracted. Several images ($N = 40$ successive frames) of the sample are acquired and the intensities $I_i$ are combined to extract a flow contrast (FC) in each pixel [Fig. 2(c)]:

$$FC = \frac{\sqrt{\sum_{i=1}^{N/2} (I_{2i} - I_{2i-1})^2}}{\sum_{i=1}^{N} (I_i)}. \qquad (1)$$

The calculated contrast is similar to the one used in laser speckle imaging.[12] Additionally, a similar motion-analysis processing has been successfully exploited by Schwerte and Pelster[6] to obtain maps of the circulatory system of zebrafish larvae.

Flow-OPT provides 3-D reconstruction by calculating flow contrast maps at several angles while rotating the sample over 360 deg. These maps are combined using a backprojection algorithmto obtain virtual sections of the circulatory system of the specimen. Image reconstruction was carried out on an eight-core processor with 32 GB RAM, and typically required 10 s on $300 \times 300 \times 300$ voxels. A threshold was applied to the sections at 10% of the maximum intensity, and a Gaussian smoothing filter with standard deviation of 2 pixels was applied in order to reduce reconstruction noise that can be caused by the stochastic noise (primarily readout noise) of the sensor.

Imaging results from the zebrafish trunk are presented in Fig. 3(a), showing a transverse section of the reconstructed vasculature. Primary arteries and veins, which are parallel to the longitudinal axis of the sample, are accurately visualized, but also capillaries as small as 10 $\mu$m can be localized. The corresponding vibrotome section at approximately the same imaging plane [in Fig. 3(b)] shows good agreement with the reconstruction. A more accurate visualization of the vessels and measurement of their size would require a reduction of artifacts still present in the reconstructed sections. High-resolution projection tomography requires that the acquired data approximate a line integral through the specimen, but this is not the case if several vessels cross each other along the line of sight from the light source to the detector, and this can give rise to streak artifacts. Additionally, we noted that the high optical absorption of certain organs (e.g., skeleton, intestine) can limit the ability of the method to detect small vessels that are close to these organs. Nevertheless, the sparsity of the vessels and the large amount of
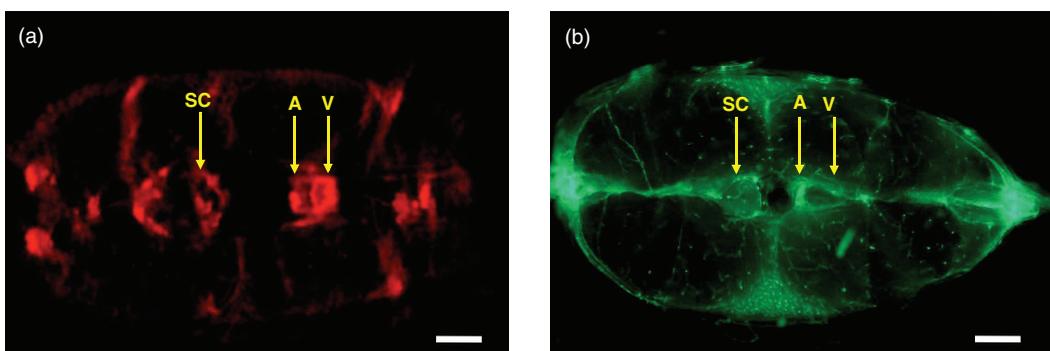


**Fig. 3** (a) Transverse section of the zebrafish in the trunk region. (b) Corresponding vibrotome section, with GFP-labeled vessels from a transgenic *tg(fli1:GFP)* juvenile fish at approximately the same plane; dorsal is to the right. (SC: spinal cord; A: aorta; V: caudal vein.) Scale bar is 100 $\mu$m.
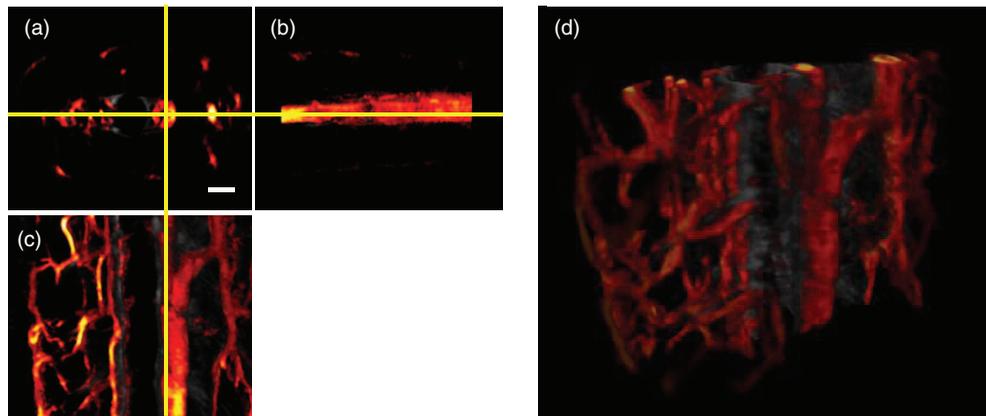
**Fig. 4** (a) Transverse, (b) coronal, and (c) sagittal sections of the juvenile zebrafish trunk. Images are in false colors; scalebar is 100 $\mu$m in all sections. (d) 3-D volume rendering (also shown in Video 2). (QuickTime, 4.8 MB) [URL: http://dx.doi.org/10.1117/1.3640808.2]

acquired projections partially compensate for these drawbacks. We expect that moving from a backprojection algorithm to algebraic reconstruction techniquesand modeling light propagation with finite elements will give a substantial improvement to the accuracy and sensitivity of the technique.

The production of a 3-D data set for the specimen means that the vascular network can be virtually sectioned along any plane and the volume can be shown in three-dimenisons. Transverse, coronal, and sagittal sections of the zebrafish trunk are shown in Fig. 4(a) and a volume rendering of the same region is shown in Fig. 4(b) and Video 2. The images are merged with brightfield-OPT reconstructions that were obtained by measuring the transmission of light through the sample at the 400 angular positions. The brightfield-OPT sections reveal the skeletal system that primarily attenuates the light propagating through the Casper zebrafish. Figure 4 demonstrates that with this technique it is possible to obtain a detailed casting of the circulatory system and, in particular, to observe the ramification of the vessels around the skeletal system. The main features of flow-OPT are: (*i*) ability to scan large volumes (up to cubic centimeter) with a relatively short acquisition time; (*ii*) low cost; (*iii*) noninvasivity; (*iv*) injection of a fluorescent probe, or the use of fluorescent reporters is not required. Additionally, we foresee that the present technique could be further enhanced to measure the speed of the bloodstream in three dimensions.

Flow-OPT is a novel *in vivo* imaging technique that could impact studies of vascular organogenesis, normal and pathological angiogenesis, and fill the existing gap in imaging techniques for larval and juvenile fish. To this end, sample immobilization protocols will be further improved and noninvasive mounting procedures will be tested in order to perform time-lapse measurements at different hours or days on the zebrafish. Technical development will include the improvement of the reconstruction algorithm and the exploitation of a fast, high-dynamic-range CMOS camera in order to reduce the measurement time and to increase imaging resolution. Finally, light scattering limits the image quality through large samples, yet the selection of ballistic photons[13] and the combination of laser speckle imaging [12] with OPT will be investigated in order to study larger, highly scattering organisms.

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