Probing Development and Molecular Function in Diffusive Living Organisms with Multispectral Optoacoustic Tomography (MSOT)

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Abstract— **Optical interrogation of biological tissues offers great variety of intrinsic probing mechanisms as well as highly specific contrast approaches based on tissue-specific expression of fluorescent proteins and extrinsically administered molecular biomarkers. Yet, most of the important living organisms and tissues remain inaccessible by the current optical imaging techniques due to complications arising from intense light scattering in tissues. In this work, a selective-plane illumination multispectral optoacoustic tomography (MSOT) technique was developed and applied for high-resolution whole-body visualization of intact mesoscopic-scale optically diffusive organisms whose sizes may vary from sub-millimeter up to a centimeter range and beyond. Utility of the method is demonstrated on several intact living organisms and small animal extremities. Furthermore, by combining multi-wavelength illumination, the method is shown capable of resolving tissue-specific expression of fluorescent proteins located deep in optically diffuse tissues.**

Keywords— **Small animal imaging, optoacoustics, photoacoustic tomography, molecular imaging**

I. Introduction

Progress in the biological sciences has often been associated with the evolution of optical imaging and the corresponding capacity to identify specific anatomical and molecular biomarkers. In particular, optical microscopy has been an essential tool for biomedical research, with applications spanning from the ability to visualize complex molecular pathways and protein function to morphological observations of development. At the organ and organism level, optical biological imaging has traditionally focused on studying life on dead specimens, i.e. through histology or immunohistochemistry, on thin sections that yield minimal photon scattering. The need to study evolution, function and disease in unperturbed environments and over time, has however entrusted modern optical visualization with the task of *in-vivo* application.

The underlying physical barrier for extending high-resolution (diffraction limited) optical imaging beyond current limits of several hundred microns is the significant light diffusion. When imaging with light through tissue, photons interact with cellular interfaces and organelles leading to multiple scattering events within the specimen under investigation [1]. The detected light therefore loses information on its origin and propagation path, blurring the images and destroying spatial resolution. Even state-of-the-art multiphoton microscopy is usually limited to superficial imaging up to a depth of 0.5-1 mm in most living tissues. Recent efforts to image entire embryos for example [2,3] require special chemical treatment of the specimen, to clear them from scattering, and are only suitable for post-mortem imaging. Macroscopic optical imaging has recently evolved as an alternative method for imaging large diffuse specimen and utilizes fully diffusive photons, typically from structures that are larger than 1 cm. In its more advanced form, techniques like Fluorescence Molecular Tomography (FMT) illuminates the sample under investigation at multiple projections and utilizes mathematical models of photon propagation in tissues, in combination with capturing diffusive photons propagating through tissue, to reconstruct the underlying imaging contrast, albeit with much lower resolution than in microscopy [1]. In contrast to microscopic three-dimensional "tissue-sectioning" imaging, *tomography* and *reconstruction* here implies the formulation of a mathematical inverse problem, whose algebraic solution (minimization) yields the reconstructed images, in analogy to methods used in X-ray CT, Single Photon Emission Tomography (SPECT) or Positron Emission Tomography (PET). Several different implementations, developed over the past years, have been successfully used to three-dimensionally image bio-distribution of fluorochromes in entire animals, molecular pathways of cancer and cardiovascular decease, offering quantitative imaging. Optical tomography in diffuse objects, however, has been developed and today applied in tissues with dimensions that are normally larger than 1 cm and usually offers low spatial resolution on the order of 1 mm. We usually refer therefore to the mesoscopic scale as one applied to organisms and tissues whose dimensions are usually between 1mm-1cm, for which neither ballistic nor diffuse photon propagation regimes apply.

Optoacoustic imaging has evolved over the last decade into a powerful modality used to investigate structural and functional information *in-vivo* noninvasively from biological specimens. Based on the optoacoustic effect, images are obtained by recording pressure waves caused by the thermal expansion that results from a small temperature rise when absorption of externally applied energy occurs. Unlike in

pure optical imaging [4], the spatial resolution here is not determined nor limited by light diffusion, therefore such performance cannot be achieved by any other optical imaging technology developed so far. Originally, optoacoustic imaging of tissues targeted endogenous tissue contrast, primarily resolving oxy- and deoxy-hemoglobin and different vascular structures [5].

Herein we present an optoacoustic tomography scanner based on multispectral selective-plane illumination [6,7]. When combined with confocal detection and specimen rotation over 360 degree projections, this technology extends imaging into living biological specimens of dimensions never optically visualized in the past. Consequently, we examine the performance of this method in imaging several important intact model organisms having no hemoglobin-based contrast. The scalability of the technology with different sizes in the mesoscopic scale is also demonstrated. In addition, we show that multispectral illumination allows visualization of spectral signatures from molecular biomarkers, like fluorescent probes and proteins, located deep in scattering living tissues. The method is therefore capable of simultaneously delivering anatomical, functional and molecular information with both high resolution and penetration capabilities.

II. Methods

The simplified scheme of the experimental setup is shown in Fig. 1. A tunable OPO laser was employed for providing multiple-wavelength illumination in the visible and the near-infrared. The pulse duration of the laser is less than 10ns and the repetition rate is 20-30Hz. The output laser beam is manipulated using cylindrical/spherical lenses, slits, and pinhole in order to adopt the incident beam shape to the size of the particular imaged object or region of interest. For detection, we used either ultrawideband PVDF transducer technology (Precision Acoustics Ltd.) or piezoelectric PZT transducers (Panametrics-NDT, Olympus) were used. The transducers are cylindrically focused in the imaging plane to allow 3D data acquisition via vertical scanning. Transducer and the 45° angled mirror are attached to a vertical translational stage, thus both the illumination and detection planes are changed simultaneously. The sample is mounted on a rotational stage located at the bottom of the water tank. In-plane data acquisition is done by 360 degrees rotation of the sample. A 14-bit resolution PCI digitizer with a sampling rate of 100 MS/s (NI PCI-5122, National Instruments Corp., Austin) is used to record the time-resolved acoustic signals detected by the transducer.

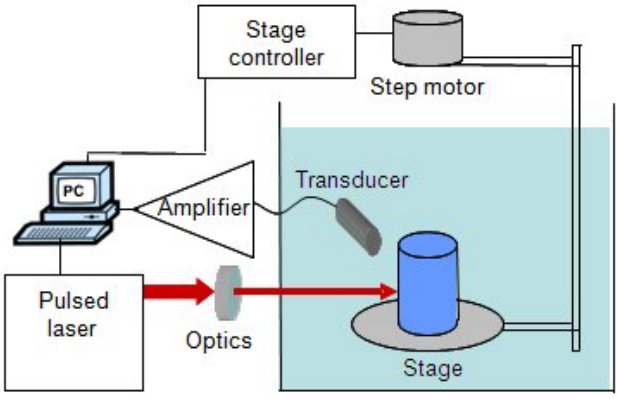

Fig. 1. Schematics of the experimental setup.

The laser, stage controllers, and data acquisition were coordinated via Labview-based interface (National Instruments Corp., Austin, TX). In addition, a photodiode (FDS010, 200-1100 nm, 1 ns rise time, Thorlabs) was placed in the vicinity of the laser output window to record the intensity change of each pulse and to normalize the detected signals for laser output instabilities. This continuous power monitoring is of critical importance for multispectral optoacoustic tomography (MSOT) reconstructions since some of important biomarkers may present only a small variation of the optical absorption over highly absorbing background, in which case even small quantification inaccuracies may lead to uninterpretable results.

III. Results

We imaged several model organisms that relate to biological discovery, whose diameter spans the mesoscopic range from 800 μm to 1 cm. Figs. 2(a)-(c) show images of developing Drosophila in its pupal stage using 750 nm selective-plane illumination The imaging planes were at two different levels – top level containing highly absorbing sensory organ of the pupa (Fig. 2(a)) and salivary glands area (Fig. 2(b)). The corresponding histological section at the salivary glands level is shown in Fig. 2(c) and shows good agreement with the reconstruction. It must be pointed out that Drosophila in its pupal stage is a fairly diffusive organism, not accessible through its intact case by microscopy techniques. By using the herein suggested method, the pupal case is readily identified in Figs. 2(b) as having rather high optical absorption as compared to the other

structures. The various fatty structures are also clearly visualized with in-plane spatial resolution on the order of 37 µm, limited by the useful bandwidth of the ultrasonic detector (up to 20Mhz). The resolution can be further improved by using ultrasonic detector of larger bandwidth.

The area containing the salivary glands is distinguishable on both optoacoustic and histological images, indicating low optical absorption properties.

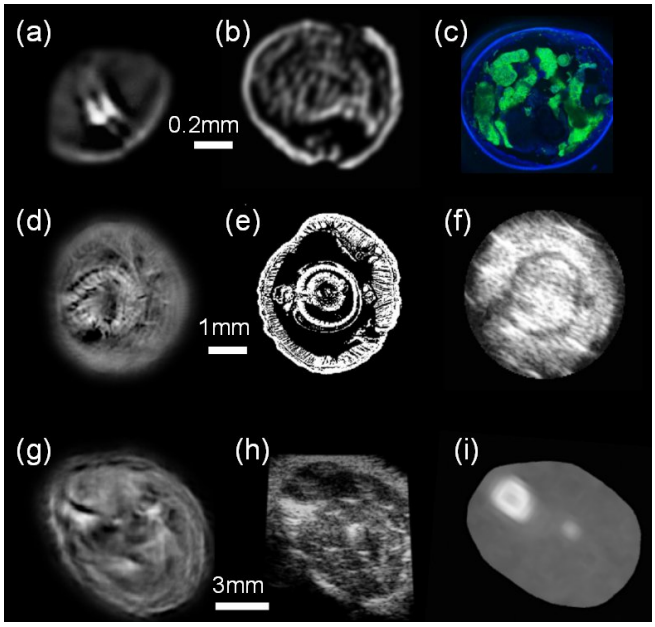

Figure 2. Images from mesoscopic-scale objects taken using selective-plane optoacoustic tomography. Cross-sectional optoacoustic images of an intact Drosophila melanogaster pupae from (a) top part containing dark-color (highly absorbing) sensory organ of the pupa; and (b) salivary glands area. (c) Histological section of the pupa at the salivary gland area (blue - dapi staining; green - GFP fluorescence expressed in the fatty structures). Images from Lumbricus Terrestris (Earthworm) are shown in (d) Selective-plane optoacoustic image; (e) Anatomical diagram; and (f) The corresponding ultrasound image acquired using high-resolution ultrasound imaging system operating at 25MHz. Images obtained from the pelvic limb of a wild-type Balb/c mouse - (g) optoacoustic tomography reconstruction; (g) The corresponding ultrasound image; (i The corresponding micro-CT image.

Fig. 2(d)-(f) shows planar images obtained from the intestine area of Lumbricus Terrestris, also well known as the Earthworm. This specie is larger in size as compared to Drosophila pupae. Although the diameter of an adult worm is usually greater than 3-4 mm, selective-plane optoacoustic imaging proved useful also in this case using the same ultrasonic transducer for detection. The reconstructed image in Fig. 2(d) provides detailed information about the inner structures (similar to histological section in Fig. 2(e)), including intestine with the folded structure of its wall, the typhlosole, muscles, dorsal and ventral blood vessels. For comparison, a pure ultrasound image of the same worm, acquired using a high-resolution ultrasound imaging system operating at 25MHz (VisualSonics Vevo 660^{TM}, VisualSonics Inc., Toronto, Ontario), failed to provide any adequate anatomical information, as evidenced from Fig. 2(f). It should be noted that the diffusion light theory is poorly applicable to the worm since the mean-free path length (MFPL) [4] occupies a considerable portion of its radius. Therefore, it is also not accessible by the diffusion optical tomography methods.

In order to demonstrate its wide scalability over different size dimensions, we applied the suggested method for imaging the pelvic limb of a wild-type Balb/c mouse. This is also a mesoscopic object whose characteristic diameter lies in the 5-10 mm range. The reconstructed cross-sectional optoacoustic image is presented in Fig. 2(g) along with the corresponding ultrasonic (Fig. 2(h)) and micro-CT (Fig. 2(i)) images, acquired approximately at the same imaging plane. Evidently, the triangular-shape *tibia* as well as the *fibula* bones are clearly visualized by all the three modalities. However, one may note that the optoacoustic image, although acquired by a much lower frequency transducer, provides enhanced contrast and other fine details that are not available in the ultrasound or CT images.

Finally, we investigate the applicability of our multispectral optoacoustic tomography (MSOT) method for whole-body imaging of fluorescent proteins. For demonstration purposes, we selected an mCherry-expressing Zebrafish as an important model organism studied extensively in the fields of neuroscience, developmental biology and genetics. Fig. 3 shows images acquired from an adult two-month old transgenic zebrafish, in which *Gal-4/UAS* system was used in order to express mCherry fluorescent reporter in the notochord. Being situated right in the middle of the fish, whose cross-sectional size in the imaging plane was about 2.5 x 4 mm, this structure is apparently not accessible by any fluorescence microscopy technique. To resolve the mCherry-marked notochord in the intact living animal, we applied imaging at three different adjacent wavelengths (587, 597 and 607 nm) lying on the steep declining slope of mCherry extinction spectra (Fig. 3(f)). This multi-wavelength illumination approach allows highly sensitive reconstruction of fluorescent proteins (having relatively rapid changes in their absorption/extinction spectra) over slowly varying tissue background absorption. Yet, since optoacoustic tomography acquires contrast from different structures surrounding the notochord, the exact distribution of the mCherry fluorescent protein cannot be distinguished on the single wavelength images (Figs. 3(a)-(c)). Spectrally-

resolved image, on the other hand, reveals the correct shape and size of the region expressing mCherry (Fig. 3(d)) with good correlation with histological section shown in Fig. 3(e).

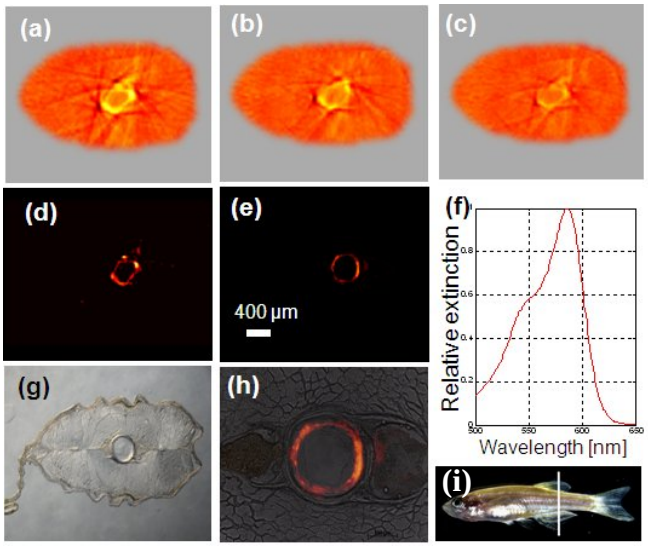

Fig. 3. Imaging of mCherry distribution in adult zebrafish. Selective-plane photoacoustic images acquired at (a) 587 nm, (b) 597 nm, and (c) 607 nm; (d) Spectrally resolved MSOT image of mCherry distribution in an intact animal; (e) Histological fluorescence image at approximately the same imaging plane (red color corresponds to mCherry-expressing notochord); (f) Extinction spectra of mCherry; (g) Histological section; (h) Overlay between histological images at the fluorescence and intrinsic channels; (i) Imaging plane.

IV. CONCLUSIONS

The ability to optically interrogate and visualize intact organisms is of high importance due to the great variety of intrinsic optical contrast and exogenous molecular probes available in the visible and near-infrared spectra. In this work, a selective-plane illumination multispectral optoacoustic tomography technique was developed and applied for high-resolution whole-body visualization of intact mesoscopic-scale optically diffusive organisms whose sizes may vary from sub-millimeter up to a centimeter range and more. The size of many relevant biological samples and model organisms, e.g. developing insects small animal extremities, animal and fish embryos as well as of some adult fishes, lie in this range. However, due to the high optical diffusion and relatively small size, they are not accessible by existing optical microscopy nor by diffusion-based optical tomography methods. Thus, selective-plane optoacoustic imaging holds promise of becoming the method of choice for imaging those organisms. Although it is known that optoacoustic imaging is sensitive to hemoglobin, good contrast was demonstrated here also for other biological tissues like fat, bones, and other internal structures.

By applying a multispectral optoacoustic tomography (MSOT) methodology, we demonstrated that other molecularly-relevant information related to biodistribution of fluorescent biomarkers and proteins, e.g. gene expression, morphogenesis, decease progression and many other targeted mechanisms, could now be visualized in whole bodies of opaque living objects with high sensitivity and spatial resolution close to a single cell dimensions.

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REFERENCES

1. Ntziachristos V, Tung C-H, Bremer C, and Weissleder R, (2002) Fluorescence molecular tomography resolves protease activity in vivo. Nat Medicin*e* 8**:** 757-760.
2. Sharpe J, Ahlgren U, Perry P et al. (2002) Optical projection tomography as a tool for 3D microscopy and gene expression studies. Science 296: 541.
3. Dodt H U, Leischner U, Schierloh A et al. (2007) Ultramicroscopy: three-dimensional visualization of neuronal networks in the whole mouse brain. Nat. Meth. 4: 331-336.
4. Vinegoni C, Pitsouli C, Razansky D, et al., (2008) Live imaging of drosophila melanogaster pupae with mesoscopic fluorescence tomography. Nat Methods 5: 45-47.
5. X. Wang, Y. Pang, G. Ku, et al. (2003) Noninvasive lasephotoacoustic tomography for structural and functional in vivo imaging of the brain Nat. Biotechnol. 21: 803.
6. Razansky D, Vinegoni C, and Ntziachristos V (2007) Multi-spectral photoacoustic imaging of fluorochromes in small animals. Opt Lett 32: 2891-2893.
7. Razansky D, Vinegoni C, and Ntziachristos V (2009) Imaging of mesoscopic-scale organisms using selective-plane optoacoustic tomography. Phys Med Biol 54.

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