Mesoscopic imaging of fluorescent proteins using multi-spectral optoacoustic tomography (MSOT)

Daniel Razansky[1,*], Claudio Vinegoni[2], and Vasilis Ntziachristos[1]

[1]Institute for Biological and Medical Imaging, Technical University of Munich and Helmholtz Center Munich, Ingolstädter Landstraße 1, 85764 Neuherberg, Germany.
[2]Center for Systems Biology, Massachusetts General Hospital and Harvard Medical School, 185 Cambridge Street, Boston, MA, 02114, USA.

ABSTRACT

Noninvasive imaging of biological tissues using visible and near-infrared light may provide numerous insights into the underlying morphology or tissue function using a great variety of contrast and probing mechanisms. Nevertheless, mesoscopic-scale (i.e 1mm-1cm sized) living organisms remain largely inaccessible by current optical imaging methods. Depending on the optical properties of a particular object, light diffusion can significantly limit the resolution that can be achieved at depths beyond several hundred microns. To enable in-vivo optical contrast imaging of many important model organisms, such as insects, worms and similarly sized biological specimens, we have developed a multi-spectral optoacoustic tomography technique for high-resolution imaging of optically diffusive organisms and tissues. The method is capable of imaging at depths from sub-millimeter up to a centimeter range with a scalable spatial resolution on the order of magnitude of a few tenths of microns. Furthermore, we show for the first time that the technique is capable of resolving spatial distribution of fluorescent proteins inside intact opaque organisms, thus overcoming depth limitations of current fluorescence microscopy techniques.

I. INTRODUCTION

In the recent years, we have assisted to a spectacular development in the field of optical microscopy, in particular for what concerns fluorescence and optical sectioning microscopies, where highly detailed 3D reconstructions of biological specimens are achieved [1-7]. The motivation for this tremendous success relies on the fact that scientific community is in great need for imaging tools that can provide insight into the biological and molecular processes within intact or living organisms, particularly for studying embryonic morphogenesis, time dependent gene expression tracking, or regulatory pathways.

Latest technical improvements allowed achieving optical sectioning microscopy with both higher resolution and penetration depths through tissues, often two contradictory aspects in imaging studies of biological tissues. When imaging through a diffusive (non-transparent) media, scattering processes lead to multiple photons scattering events within the sample under investigation due to the photons' elastic collisions with the different cellular components such as the cell membranes and the organelles. The light will therefore get diffused and the resulting image will appear blurred,

* Electronic mail: daniel.razansky@helmholtz-muenchen.de

Photons Plus Ultrasound: Imaging and Sensing 2009, edited by Alexander A. Oraevsky, Lihong V. Wang,
Proc. of SPIE Vol. 7177, 71770D · © 2009 SPIE · CCC code: 1605-7422/09/$18 · doi: 10.1117/12.810860

preventing the visualization and resolution of deep structures and limiting the maximum penetration depth to the order of one transport mean free path-length (MFPL) in tissue [8]. There are many methods to discriminate between diffused and ballistic or near-ballistic photons, considering only the last ones as useful for imaging purposes. These are exploited in e.g. confocal microscopy [5]. Nonlinear techniques like multiphoton microscopy are less sensitive to scattering and make use of a localized nonlinear signal generation due to high-order light matter interactions [6,7]. Nevertheless, all these techniques do not allow imaging through non-transparent (diffusive) samples with sizes on the order or bigger than 100's of microns.

On the other hand, some novel volumetric optical imaging techniques that account for the diffusive propagation of photons in tissues, were recently proposed and demonstrated three-dimensionally molecular imaging in deep tissues of small animals in-vivo [8,9]. Unfortunately, the compromise of working with diffusive photons is reduced spatial resolution, usually in the millimeter range, depending on the optical scattering characteristics of the sample under investigation, imaging depth, and equipment-related limitations. In addition, image reconstruction based on light diffusion theory is usually limited to samples greater than 10 MFPL, thus typically not suitable for imaging samples smaller than 1 cm.

More recently, alternative optical approaches such as optical projection tomography (OPT) or selective plane illumination microscopy (SPIM) [10-12] made an attempt to overcome the diffusion problem by operating post-mortem on chemically treated embryos in order to reduce the scattering and to increase specimen's transparency. While this could in fact lead to striking optical sectioning images with resolution limited only by the optics used, the two techniques are still not appropriate for imaging intact organisms, except for cases with natural transparency, occurring e.g. in fishes' embryos.

As a result of the above limitations, an imaging gap exists between optical microscopy and state-of the-art optical diffusion tomography methods for in-vivo imaging. Therefore, there is a high demand for a light-based imaging method in non-transparent intact samples in the optical mesoscopy range beyond the 1 MFPL limit. This will allow visualization of developing insects, animal embryos or small animal extremities, while retaining high intrinsic optical contrast and sensitivity to numerous exogenous contrast agents and stimuli.

In this work we investigate the ability of optoacoustic tomography to provide visualization of mesoscopic scale living samples and organisms. . It was demonstrated that optoacoustics is capable of having both high spatial resolution, attributed with scattering-free ultrasonic imaging, and high contrast, associated with large differences in optical absorption of tissues [8], [13-15]. Recently, biomedical optoacoustic tomography was successfully applied to various diagnostic imaging areas, i.e. breast cancer detection, skin microscopy, small animal and vascular imaging [13-17] as well as detecting fluorescent molecular probes [18] and probing gene-expression in mice *in-vivo* using chromogenic assays [19]. Most of the studies, however, focused on highly vascularized human and mouse tissues, relying on high optical absorption contrast of blood as compared to other less absorbing tissues.

Herein we demonstrate that, using an appropriate illumination and detection schemes, optoacoustic imaging can successfully be extended to a wide range of small animal species and organisms. We further show that multi-spectral optoacoustic tomography is capable of visualizing fluorescent proteins in an intact Drosophila pupa, an important model organism presenting significant scattering and not accessible by the exisiting fluorescence microscopy techniques.

II. METHODS

For tomographic multi-spectral acquisition, we used an OPO laser (Model MOPO, Spectra Physics, Mountain View, CA), tunable throughout the visible and near-infrared regions. The imaged objects were placed on a rotation stage, controlled via stage controller (SC, ESP-3000, Newport Corp., Irvine, CA). The laser beam is passed through a cylindrical lens and directed upon the sample submerged into water. Cylindrically-focused broadband ultrasonic transducers (Model V319, Panametrics), with central frequencies of 15Mhz, was used to record the optoacoustic signals transmitted from the imaged objects. The recorded time-resolved signals were amplified, digitized, and averaged by an embedded oscilloscope PCI card at 100 Msps (NI PCI-5122, National Instruments Corp., Austin, TX) with 14-bits

vertical resolution. The samples were rotated 360° with 3° steps in order to enable in-plane 2D image reconstruction using filtered backprojection algorithm [20].

The principle of fluorescent protein detection is based on differentiation of the absorption spectral signature of the protein over the background tissue absorption by spectroscopic analysis of optoacoustic data acquired at multiple wavelengths [18].

III. RESULTS

We imaged several objects, whose diameter spans the mesoscopic range. Fig. 1 shows optoacoustic tomography images obtained from an Earthworm (Lumbricus Terrestris). These species are large in size (diameter of an adult worm is usually greater than 3-4 mm) and very diffusive. Nevertheless, our approach was proved useful and the reconstructed image in Fig. 1(a) provides detailed information about the inner structures of the worm, including gizzard, intestine, the folded structure of its wall, dorsal and ventral blood vessels. For comparison, a pure ultrasound image of the same worm, acquired using VisualSonics Vevo 660^{TM} (VisualSonics Inc., Toronto, Ontario) high-resolution ultrasound imaging system operating at 25MHz, fails to provide any adequate anatomical information, as evidenced from Fig. 1(b).

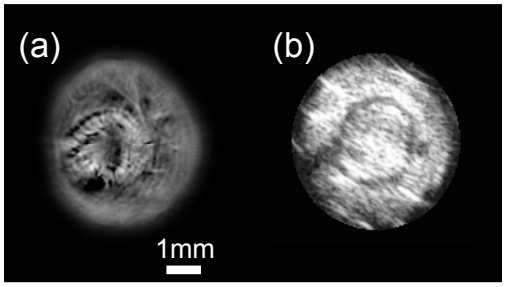

Fig. 1. Cross-sectional images of Lumbricus Terrestris. (a) Optoacoustic image made at 750 nm; (b) The corresponding ultrasound image acquired using high-resolution ultrasound imaging system operating at 25MHz.

Fig. 2 shows multispectral optoacoustic tomography images of developing Drosophila (fruit fly) in its pupal stage, in which eGFP fluorescent protein was expressed in the salivary glands. The imaging plane was located in the bottom part of the glands area. It must be pointed out that Drosophila in its pupal stage is a fairly optically diffusive organism, not accessible by any microscopy technique [21]. Indeed, optoacoustic tomography resolved several anatomical structures within the pupa. For instance, the pupa case is readily identified in the single wavelength images (Figs. 2(b)-(d)) as having rather high optical absorption as compared to the other structures. The various fatty structures are also clearly visualized with the smallest resolvable structure on the order of 40 μm, limited by the useful bandwidth of the 15 Mhz ultrasonic detector.

Finally, we investigate the applicability of our method for whole-body imaging of fluorescent proteins. To resolve the eGFP-marked salivary glands in the intact living animal, we applied imaging at three different adjacent wavelengths (488, 498 and 508 nm) lying on the steep declining slope of GFP extinction spectra (Fig. 2(a)). 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. Apparently, the single wavelength images in Fig. 2(b)-(d) provide no definitive information regarding the location of fluorescent proteins. Nevertheless, by

multispectral fitting of image intensities with GFP spectral curve in Fig. 2(a) on a per-pixel basis, we were readily able to accurately resolve the fluorescence proteins expressed in the salivary glands, as demonstrated in Figs. 2(e) and (f). The corresponding histology is shown in Fig. 2(g).

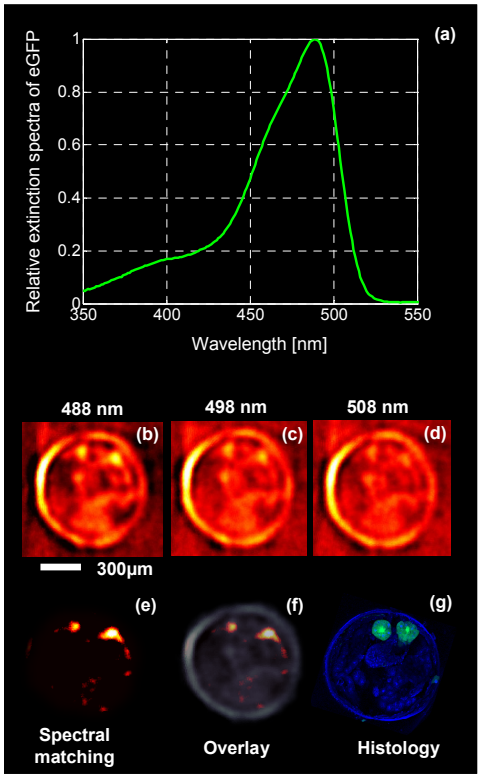

Fig. 5. Multispectral optoacoustic imaging of fluorescent protein distribution in Drosophila melanogaster pupa. (a) Extinction spectra of eGFP; Single wavelength optoacoustic images acquired at (b) 488 nm, (c) 498 nm, and (d) 508 nm; (e) Spectrally resolved image of eGFP using the three wavelengths; (f) Overlay between the image at 488 nm and the spectrally-resolved image; (f) corresponding histology of DAPI-stained pupa at approximately the same imaging plane (green color corresponds to GFP-expressing salivary glands).

IV. DISCUSSION AND CONCLUSIONS

The ability to optically interrogate and visualize an intact organism is of high importance due to the great variety of intrinsic optical contrast and exogenous molecular beacons available in the visible and near-infrared spectra. In this work, a selective-plane illumination optoacoustic tomography technique with confocal acoustic detection 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. 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 any of the existing optical microscopy or diffusion optical tomography methods. Thus, the suggested method is holding the promise of becoming the ultimate choice for imaging those organisms. Although it is well known that optoacoustic

imaging is normally mostly sensitive to hemoglobin-containing substances like whole blood, a good contrast was demonstrated here also for other biological tissues.

By applying the multispectral imaging methodology, we also demonstrated that other molecularly-relevant information related to bio-distribution of fluorescent proteins, e.g. gene expression, morphogenesis, decease progression and many other targeted mechanisms, could now be visualized in whole bodies of living mesoscopic objects with high sensitivity and spatial resolution. For mesoscopic sized objects, the scattering causes only partially widening of the illumination beam as it goes through the object. Therefore, with our method, only the imaged planes are preferably illuminated and problems related to the bleaching of the fluorescent proteins, that imply a reduction in their absorption, are dramatically reduced. This would be particularly useful for 3D tomographic reconstructions, where a selective plane illumination strategy will benefit over a whole body illumination.

REFERENCES

[1] Giepmans B. N. G., Adams S. R., Ellisman M. H. and Tsien R. Y., "The fluorescent toolbox for assessing protein location and function," *Science* 312, 217 (2006).

[2] Lichtman J. W. and Conchello J. A., "Fluorescence microscopy," *Nat. Methods* 2(12), 910 (2005).

[3] Conchello J. A. and Lichtman J. W., "Optical sectioning microscopy," *Nat Methods* 2(12), 920 (2005).

[4] Bahlmann K., So P. T. C., Kirber M., Reich R., Kosicki B., McGonagle W. and Bellve K., "Multifocal multiphoton microscopy (MMM) at a frame rate beyond 600 Hz," *Opt. Express* 15(17), 10991 (2007).

[5] Minsky M., *US Patent* 3,013,467 (1961).

[6] Denk W., Strickler J. H. and Webb W. W., "2-photon laser scanning fluorescence microscopy," *Science* 248 (4951), 73 (1990).

[7] Helmchen F. and Denk W., "Deep tissue two-photon microscopy," *Nat. Methods* 2(12), 932 (2005).

[8] Ntziachristos V., Ripoll J., Wang L. H. V. and Weissleder R., "Looking and listening to light: the evolution of whole-body photonic imaging," *Nat. Biotechnol.* 23 (3), 313 (2005).

[9] Ntziachristos V., Tung C-H, Bremer C. and Weissleder R., "Fluorescence molecular tomography resolves protease activity in-vivo," *Nat. Med.* 8(7), 757-760 (2002).

[10] Sharpe J., Ahlgren U., Perry P. et al., "Optical projection tomography as a tool for 3D microscopy and gene expression studies," *Science* 296 (5567), 541 (2002).

[11] Huisken J., Swoger J., Del Bene F. et al., "Optical sectioning deep inside live embryos by selective plane illumination microscopy," *Science* 305 (5686), 1007 (2004).

[12] Dodt H. U., Leischner U., Schierloh A. et al., "Ultramicroscopy: three-dimensional visualization of neuronal networks in the whole mouse brain," *Nat Methods* 4(4), 331-336 (2007).

[13] Hoelen C. G. A., de Mul F. F. M., Pongers R. and Dekker A., "Three-dimensional photoacoustic imaging of blood vessels in tissue," *Opt. Lett*. 23(8), (1998).

[14] Wang X., Pang Y., Ku G., Xie X., Stoica G., Wang L. V., "Noninvasive laser-induced photoacoustic tomography for structural and functional in vivo imaging of the brain," Nat Biotechnol. 21, 803-806 (2003).

[15] Kruger R. A., Kiser W. L., Reinecke D. R., Kruger G. A., Miller K. D., "Thermoacoustic molecular imaging of small animals," Mol. Imag. 2,113-123 (2003).

[16] Zhang E. Z., Laufer J., Pedley R. B. and Beard P., “3D photoacoustic imaging system for in vivo studies of small animal models,” Proc. SPIE 6856, 68560P (2008).

[17] Zhang H. F., Maslov K., Stoica G. and Wang L V.,"Functional photoacoustic microscopy for high-resolution and noninvasive *in vivo* imaging," *Nat. Biotechnol.* 24, 848-851 (2006).

[18] Razansky D., Vinegoni C. and Ntziachristos V., "Multispectral photoacoustic imaging of fluorochromes in small animals," *Opt. Lett.* 32(19), 2891-2893 (2007).

[19] Li L., Zemp R. J., Lungu G., Stoica G. and Wang L. V., "Photoacoustic imaging of LacZ gene expression *in vivo,*" *J. Biomed. Opt.* 12, 020504 (2007).

[20]Razansky D. and Ntziachristos V., "Hybrid photo-acoustic fluorescence molecular tomography using finite-element-based inversion," *Med. Phys.* 34(11), 4293-4301 (2007).

[21] Vinegoni C., Pitsouli C., Razansky D., Perrimon N. and Ntziachristos V., "In vivo imaging of Drosophila melanogaster pupae with mesoscopic fluorescence tomography," *Nat. Methods* 5(1), 45-47 (2008).