Fluorescent protein imaging with multispectral optoacoustic tomography

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ABSTRACT

In this work, we have developed a selective-plane illumination multispectral optoacoustic tomography (MSOT) technique for high-resolution whole-body visualization of intact optically diffusive organisms whose sizes may vary from sub-millimeter up to a centimeter range and beyond. By combining multi-wavelength illumination, the method is shown capable of resolving tissue-specific expression of fluorescent proteins and other molecular biomarkers located deep in living optically diffuse tissues.

Keywords: Fluorescent proteins, optoacoustics, molecular imaging, small animals, photoacoustic tomography

I. INTRODUCTION

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.

More recently, alternative optical approaches such as optical projection tomography (OPT) or selective plane illumination microscopy (SPIM) [1] 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.

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 [2]. 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 mean-free-path-lengths (MFPL), thus typically not suitable for imaging samples smaller than 1 cm.

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II. METHODS

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 selective-plane optoacoustic tomography technique for high-resolution imaging of optically diffusive organisms and tissues.

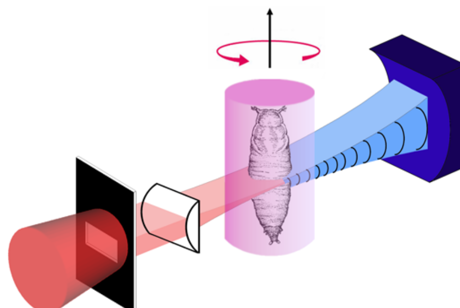

Figure 1. Schematics of the selective-plane optoacoustic tomography technique.

The experimental setup (Fig. 1) utilizes nanosecond pulsed laser illumination, which is passed through a variable slit aperture and focused, using a cylindrically focusing lens, onto the sample, creating planar sheet of light. The imaged objects are fixed on the rotation stage that is submerged into water to facilitate detection of acoustic signals. Optoacoustic signals are recorded by a broadband ultrasonic transducer, cylindrically focused at the optical illumination plane (confocal arrangement). The focal line was usually extended for about 1 MFPL into the scattering object as to allow maximal possible confinement of the planar light's sheet within the acoustic detection plane (image plane). As many organisms may present particularly high absorption contrast between different structures, this kind of confocal illumination-detection strategy is especially useful in the cases studied here. This is because the light is only partially diffused as it passes through the mesoscopic-scale objects (diameters up to few mm), thus signals coming from "out-of-focus plane" absorptive structures are minimized.

In order to prove the ability of the method to visualize common optical molecular and genetic markers, such as fluorescent proteins, multispectral optoacoustic tomography (MSOT) is used that records optoacoustic data at multiple wavelengths [3]. 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. For instance, in the case of mCherry fluorescent protein, the extinction (absorption) spectrum of the molecule will exhibit sharp resonance around 585 nm (as shown in Fig. 2).

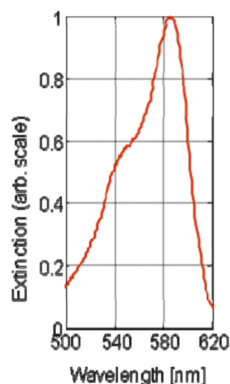

Figure 2. Relative extinction of mCherry fluorescent protein.

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The location and concentration of the fluorescent protein in the imaged volume can be resolved by spectral processing of opto-acoustic images obtained at n discrete wavelengths $\lambda_1,...,\lambda_N$ around the peak extinction of the protein. To improve accuracy, optoacoustic measurements can be further taken in control animals containing no fluorescent proteins in order to establish spectral behavior of the background absorption. Subsequently, it is assumed that every pixel k in the optoacoustic image represents a combined contribution of the fluorescent protein and the background. This can be written in the form of *N* linear equations :

$$\mu_a^k(\lambda_m) = \alpha_b(\lambda_m)c_b^k + \alpha_{FP}(\lambda_m)c_{FP}^k, \quad m = 1,...,N,$$

where $\mu_a^k(\lambda)$ is the reconstructed wavelength-dependent absorption in pixel *k*, $\alpha_b(\lambda)$ and $\alpha_{FP}(\lambda)$ are the molar extinction spectra of the FP and the background, and c_b^k and c_{FP}^k are the corresponding concentrations. Using the measured total absorption values and the known spectra for the measured wavelengths, the concentrations c_{FP}^k of the fluorescent protein and the background c_b^k are subsequently reconstructed from the above linear equations on a per-pixel basis using linear regression method. It should be noted that, while $\alpha_{FP}(\lambda)$ and c_{FP}^k represent the actual molar extinction coefficient of the fluorescent protein and its concentration, the measured background spectra $\alpha_b(\lambda)$ and the extracted values of the concentration c_b^k have an arbitrary scale values and only their product $\alpha_b(\lambda)c_b^k$ has a real physical interpretation. Additional spectral contributions can be introduced by simply adding new terms to the spectral equations.

III. RESULTS

To investigate the *in-vivo* capacity of the method to image beyond the limits of modern microscopy, adult zebrafish was selected as imaging target, also because this organism is extensively used in various fields of modern biology. In this way, by extending non-invasive imaging during optically transparent embryogenesis to opaque stages of this model organism, we hope to open novel avenues in numerous fields of genetic research, such as investigating molecular and cellular mechanisms of disease etiology and progression, tumorigenesis and metastasis formation or aging.

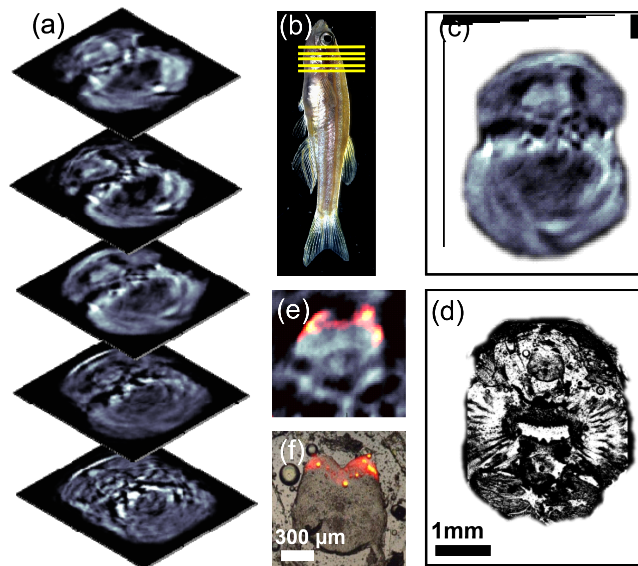

Figure 3. Multispectral optoacoustic tomography attains whole-body visualization of deep-seated fluorescent protein molecules in adult Zebrafish with high (mesoscopic) resolution (currently on the order of 38 microns), while simultaneously providing the necessary reference anatomical images. The images were three-dimensionally (3D) acquired *in-vivo* through the brain of an adult (6 months old) mCherry-expressing transgenic Zebrafish with a cross-section diameter of around 6 mm. The results demonstrate ability to reveal many high-resolution morphological features, as evident from (a) and (c), supported by the corresponding histology (d). Moreover, multispectral reconstructions also accurately resolve FP expression in the brain of an intact living animal (e), in high congruence with the corresponding epi-fluorescence images of the dissected fish at the hindbrain level (f).

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We used transgenic six-month old zebrafish, in which the *Gal-4/UAS* system was utilized to express mCherry fluorescent protein in the hindbrain under control of the zic1/4 enhancer. To showcase the MSOT capacity of performing three-dimensional (3D) *in-vivo* scans we have imaged through the head of an intact fish (cross-section diameter of around 6 mm, Fig. 3b). The imaging results, shown in Fig. 3, demonstrate ability to reveal many morphological features, as evident from 3D morphological image stack acquired at 585 nm (Figs. 3a, c), supported by the corresponding histology (Fig. 3d). Moreover, multispectral reconstructions accurately resolve FP expression in the brain, in the dorsal-most region of the anterior hindbrain (the crista cerebellaris), of an intact living animal (Fig. 3e), in high congruence with the corresponding epi-fluorescence images of the dissected brain (Fig. 3f).

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, showcased here by imaging of the adult Zebrafish. Although it is well known that optoacoustic imaging is normally mostly sensitive to hemoglobin-containing substances like whole blood, a good contrast is demonstrated here also for other tissues.

By applying the multispectral optoacoustic imaging methodology, the MSOT, we also demonstrate 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 adult Zebrafish 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 is particularly useful for 3D tomographic reconstructions, where a selective plane illumination strategy benefits over a whole body illumination.

REFERENCES

[1] H. U. Dodt, U. Leischner, A. Schierloh et al., "Ultramicroscopy: three-dimensional visualization of neuronal networks in the whole mouse brain," *Nat. Meth.* 4: 331-336 (2007).
[2] V. Ntziachristos, J. Ripoll, L. V. Wang, and R. Weissleder, "Looking and listening to light: the evolution of whole-body photonic imaging," *Nat. Biotechnol.* 23, 313 (2005).
[3] D. Razansky, M. Distel, C. Vinegoni, R. Ma, N. Perrimon, R. W. Köster, and V. Ntziachristos, "Multi-spectral optoacoustic tomography of deep-seated fluorescent proteins *in-vivo,*" *Nature Phot.* 3(7), 412-417 (2009).

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