Optical Projection Tomography for In-Vivo Imaging of *Drosophila melanogaster*

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BIOGRAPHY

Heiko Meyer received diplomas in applied laser technology at the University of Applied Sciences, Emden, Germany, and in medical product development from the Hanzehogeschool van Groningen, The Netherlands. He started his PhD in 2004 at FORTH in Crete, Greece. He is now with the Biophotonics Group at the Laser Zentrum Hannover, Germany, continuing his research in tomographic imaging and entering new fields such as ultrashort pulse cell manipulation and imaging.

ABSTRACT

Optical projection tomography is a recently developed tomographic technique used to acquire 3D microscopy data from specimens 1-10 mm thick. In previous work, to reduce scattering and attenuation the specimens were fixed and cleared, but clearing makes in-vivo fluorescence OPT of the sample impossible. We present a novel OPT technique for obtaining 3D images of both anatomy and fluorescent-protein expression *in vivo*, and we apply these techniques to gene expression mapping of *Drosophila melanogaster* development. We believe this approach will prove useful for in-vivo follow-up measurements of gene expression patterns in mm-sized samples, traditionally unavailable when using imaging techniques such as confocal microscopy.

KEYWORDS

optical projection tomography, fluorescence microscopy, three dimensional imaging , in vivo

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INTRODUCTION

In the field of microscopy, spatial in-vivo imaging techniques have developed rapidly within the last decade [4, 5]. Most of these new techniques are based on optical sectioning techniques such as confocal microscopy [6]. Other in-vivo techniques also exist: some of the commonly used contrast enhancing techniques are differential interference contrast microscopy (DICM)[7, 8], and optical coherence microscopy and tomography (OCM and OCT) [9, 10]. Despite being very popular techniques in the field of 3D microscopy, most of these techniques come with significant limitations when it comes to high-resolution whole specimen imaging and/or imaging of multiple contrast agents or fluorescent proteins.

Recently, several new microscopical imaging techniques have been developed that offer an alternative to serial sectioning of samples by making use of tomographic approaches with visible light. Techniques of particular interest are optical projection tomography (OPT) [1] and selective plane illumination microscopy (SPIM) [11, 12].

OPT has shown great potential in high resolution tomographic imaging, employing simple and cost effective setups with the capacity for fluorescence as well as absorption imaging [2, 3]. Eliminating the need for serial sectioning of the specimen, OPT makes use of filtered back projections of images acquired of a specimen imaged at a number of equidistant angles. The filtered backprojection approach (FBP) is based on the inverse radon transform theory originally developed for X-ray tomography. OPT can offer magnification dependant resolution down to 1-5 µm [13-15].

Over the past five years, OPT has been applied to imaging of embryonic development [2], fluorescent immunohistochemistry [16], gene expression imaging [17], transgenic imaging [18] and phenotyping [19] in various tissues and organisms [20-22]. For the in-vivo purposes of this paper, it is important to note that in all the cases listed above, apart from Lee et al. [20] where in-vivo imaging in plant development was presented, in-vivo measurements were not performed due to the employment of sample clearing techniques. These techniques involve clearing the sample tissue with a 1:2 mixture of benzyl alcohol and benzyl benzoate (BABB, Murray's clear) [1, 23]. Even though the sample clearing approach gives the best results in terms of resolution and sensitivity, the aggressive nature of the organic solvent renders in-vivo imaging of cleared specimens impossible. The same problem exists with the imaging of fluorescent proteins.

Another application of OPT has been demonstrated in single-cell imaging achieving sub-micrometre resolution in reconstructed images as well as in the rendered data [24]. This level of resolution is achieved using not only normal OPT projections but also the microscopes ability of scanning through the specimen to create so called 'pseudoprojections'. It has also been shown that fixed and labelled imaging in murine organs is also feasible using OPT [25, 26].

In this article we describe a custom optical

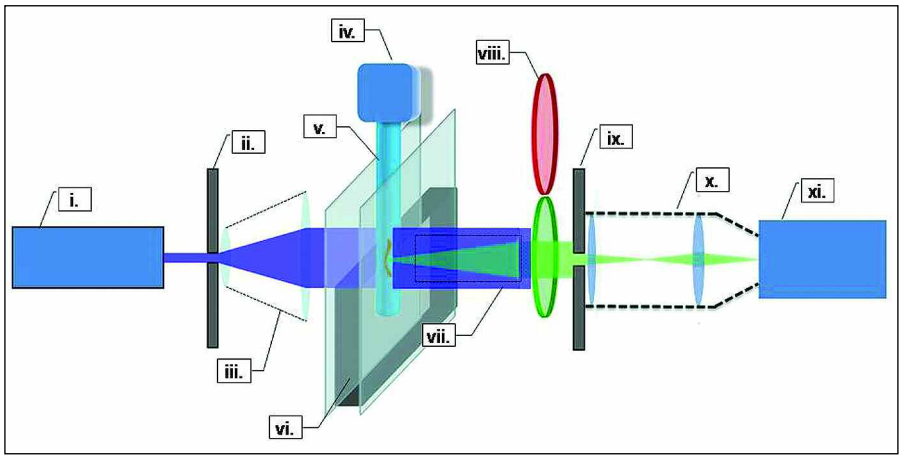

Figure 1:
Schematic of the optical projection tomography microscope setup. [i] laser, [ii] shutter, [iii] beam expander, [iv] stepper motor, [v] capillary mount with micro capillary, [vi] index matching vessel, [vii] microscope objective, [viii] filter slide with fluorescence filters, [ix] variable aperture, [x] lens tube system, [xi] ICCD camera.

projection tomography setup and show its in-vivo imaging capacity with *Drosophila melanogaster* expressing green fluorescent protein (GFP). To our knowledge, this is the first time that 3D in-vivo imaging of millimetre-sized specimens such as *Drosophila melanogaster* has been reported using OPT. We would like to re-emphasize that this OPT study using fluorescent protein-expressing transgenics could not be performed using BABB for specimen clearing as reported in other OPT papers.

MATERIALS AND METHODS

Experimental Setup

The experimental setup is depicted in Figure 1 and employs two DPSS Lasers (100 mW cw emitting at 473 nm and 30 mW cw emitting at 532 nm, Laserlight, Berlin, Germany) (labelled [i] in Figure 1). A fast laser shutter (LS 6, UniBlitz, Rochester, NY) [ii], and a 5× beam expander (Edmund Optics Inc., Karlsruhe, Germany) [iii] are used to control the duration and pattern of excitation, enlarging the beam diameter from 2 mm to 5 mm so as to fully illuminate samples without the need for scanning. The sample holder employs a stepper motor (Oriental Motors) [iv] capable of up to 500 steps per revolution and a custom made capillary holder that transmits the rotation of the motor to a range of single-use microcapillary tubes of refractive index n_D = 1.474 at 589 nm (Blaubrand - intraMARK, BRAND, Wertheim, Germany). The different sizes of capillary tubes available have inner diameters of 0.29 mm (1-5 µl), 0.58 mm (20 µl), 0.91 mm (50 µl) and 1.19 mm (100 µl). One end of the capillary is immersed in a custom made index matching vessel [vi] using 50×24×0.15 mm borosilicate cover slips (n_D = 1.474 at 589 nm) containing 87% glycerol solution as an index matching fluid (n_D = 1.47 at 589 nm) to minimize internal reflection and refraction of the excitation and emission light.

The imaging unit consists of a lens tube system (InfiniTube, Infinity, Boulder, CO) [x] incorporating a custom filter slide [viii] for 25 mm diameter fluorescence filters (525±17.5 nm for GFP, 593±20 nm for DsRed, both Shamrock, Rochester NY). Two infinity-corrected microscope objectives are available at 5× and 10× magnification [vi] (Mitutoyo, Kawasaki, Japan). Images are focused onto a thermoelectrically cooled intensified CCD with 1002×1004 pixel resolution [xi] (DV885, Andor Technology, Belfast, Northern Ireland). The depth of field of the system can be selected using a variable aperture [ix] positioned behind the microscope objective[14].

In-vivo Specimen Preparation

In previously published OPT results on specimens on the millimetre scale [1-3, 27], BABB was used to decrease the opacity of the sample. This reduces scattering and attenuation, thereby easing signal acquisition, but also unfortunately precludes in-vivo imaging. Since our goal is in-vivo OPT imaging, alternative procedures for sample preparation were investigated. Our technique is to immerse samples in an 87% glycerol solution (FLUKA, Sigma Aldrich). This approach results in two advantages, the first being that the close index match between the glycerol solution, the capillary tube and the walls of the index matching vessel reduces refraction, the second being that samples such as *Drosophila melanogaster* pupae have been shown to survive for at least three hours under these conditions [28], thereby allowing time for in-vivo imaging, since the whole duration of the measurements is typically 5 minutes per channel. For specimens in the larval stage a 1-1.5% agarose solution containing 20 mM levamisole for immobilization is used. The agarose solution results in an index match still suitable for imaging. The specimen holders have been chosen to match typical specimen dimensions.

Available are four different sizes of micro capillaries with various diameters as described above. In case smaller dimensions are needed, the 1-5 µl capillaries can be resized to smaller diameters using a micropipette puller (Model PN-30, Narishige Co., Tokyo, Japan).

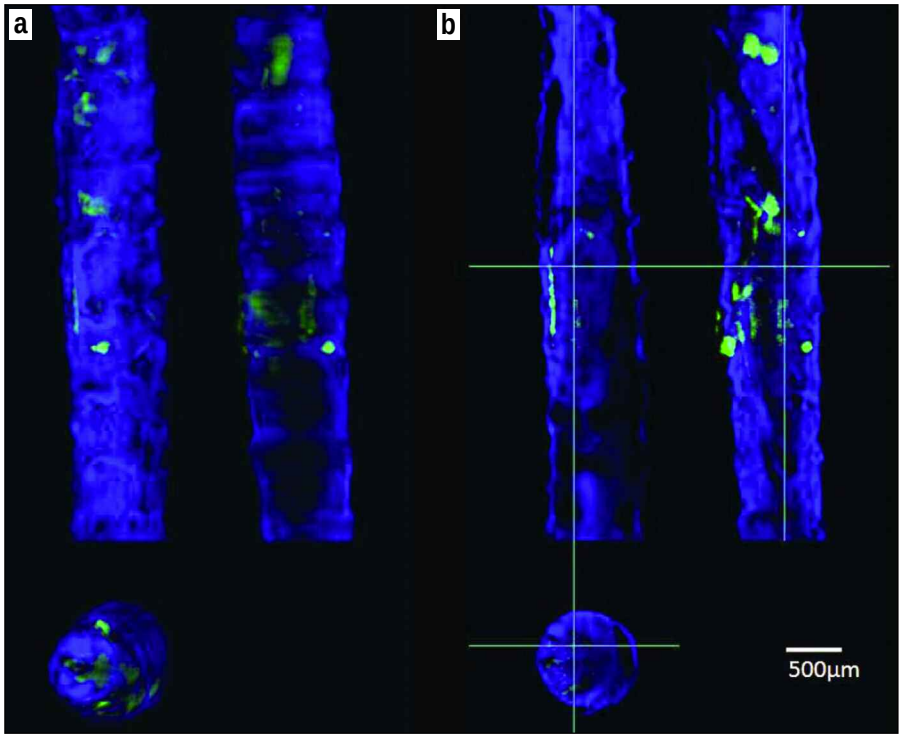

Figure 2:
Overlaid reconstruction of the white light (blue) and fluorescence (green) data taken in-vivo from a day 5 stage Drosophila melanogaster larva (MExT3e). (a) 3D overlay. (b) Internal sections longitudinal and transversal through the specimen.

Data Acquisition and Image Reconstruction

Data acquisition was conducted using custom software coded in LabView and Matlab. The software controls the camera, the stepper motor and the laser shutter, and runs on a dedicated 3.6 GHz PC with 2 GB RAM. In fluorescence mode, the laser shutter is controlled so as to eliminate the sample only during image acquisition in order to minimize fluorescence bleaching. This reduces the need to account for bleaching artifacts during the reconstruction procedure [13].

Image reconstruction is performed using the FBP algorithm employing a Hamming filter [29]. The centre of rotation is found making use of the variance in reconstructions, as shown in [13]. Data acquired normally consist of 500 projections equally spaced over 360º, resulting in angle steps of 0.72º. Data sets are recorded for three channels: fluorescence, excitation and white light illumination. The white light illuminated images offer anatomical information which aids visualization of the fluorescence data. In all cases, light is collected in transmission mode (see Figure 1). 2×2 CCD pixel binning was used to reduce acquisition time, further reduce bleaching effects and also enable faster data reconstruction.

RESULTS

During testing of the OPT setup, we performed a series of OPT experiments on *Drosophila melanogaster* at different stages of development and targeting different expression patterns. In particular, we imaged at the larvae, pupa and adult stages with different expression patterns. The results from the pupa and larval stages are presented in this section.

Imaging of GFP Expression in the Brain at the Larval Stage

Figure 2 shows reconstructions of the fluorescence distribution of a Minos-based exon trap line (MExT3e, A. Metaxakis and C. Savakis, unpublished data) in a *Drosophila* larva. The main expression pattern is found in the larval brain but there is also significant autofluorescence detected in the gut.

Imaging of Salivary Glands at Pupa Stage

Figure 3 shows the in-vivo results from an early stage (4-5th day) pupa of the *Drosophila melanogaster* enhancer trap line GR2x. The Gal4 / UAS system drives the expression of the

GFP protein at the salivary glands during the larvae and early pupa stages [30]. The results show the reconstructed superposition of the GFP fluorescence in the salivary glands and the anatomy acquired with white light illumination (blue). The salivary glands are visible as green areas of fluorescence. Figure 2a shows the volumetric information of the whole specimen, while Figure 2b represents the internal sections dorsolateral and saggital through the specimen.

DISCUSSION AND FUTURE OUTLOOK

In this article we have demonstrated the in-vivo optical projection tomographic imaging of the salivary glands and the brain of *Drosophila melanogaster* in the larva and the pupa stage. Additionally, and in a more general sense, we have reported the capability of an OPT-based setup not only in measuring fluorescence patterns in cleared ex-vivo samples but also in opaque and low-scattering tissues, which enables in-vivo imaging in specimens with tissue properties, which, so far, has only been contrived using mesoscopic fluorescence tomography [31].

OPT can be used to study morphometry during the developmental stages delivering high (~5 µm) spatial resolution not only for cleared specimens but also living opaque organisms. With the addition of fluorescence imaging, OPT allows studies of fluorescence distribution *in vivo*.

Another approach towards the quantification of fluorescence data using OTP is the implementation of the properties of light propagation from a point source, since point sources lying different distances from the objective plane will be imaged with different intensities thus giving false quantitative values [32]. This application will be of great advantage towards quantitative imaging using optical projection tomography.

All results presented in this paper were produced by the filtered backprojection of 500 projections. It is well-known that reconstruction images of similar quality can be obtained from a smaller number of projections using other iterative reconstruction algorithms such as the algebraic reconstruction technique [28] which offers the opportunity for a fuller model of light propagation. We are currently working towards this in order to greatly reduce the number of projections required for equivalent reconstructions, thereby reducing the total time required for the completion of an OPT imaging experiment.

In principle, with very few projections and making use of more detection points (CCD cameras) or mirrors, it should be possible to obtain real-time 3D data acquisition of live samples.

Another planned improvement to the setup is a rapid filter-changing mechanism that would allow multi-colour imaging of the same specimen within the same experiment. Such an advance would allow the tracking of area-area interaction or even cell-cell interaction *in vivo* which could also be useful in cell migration studies.

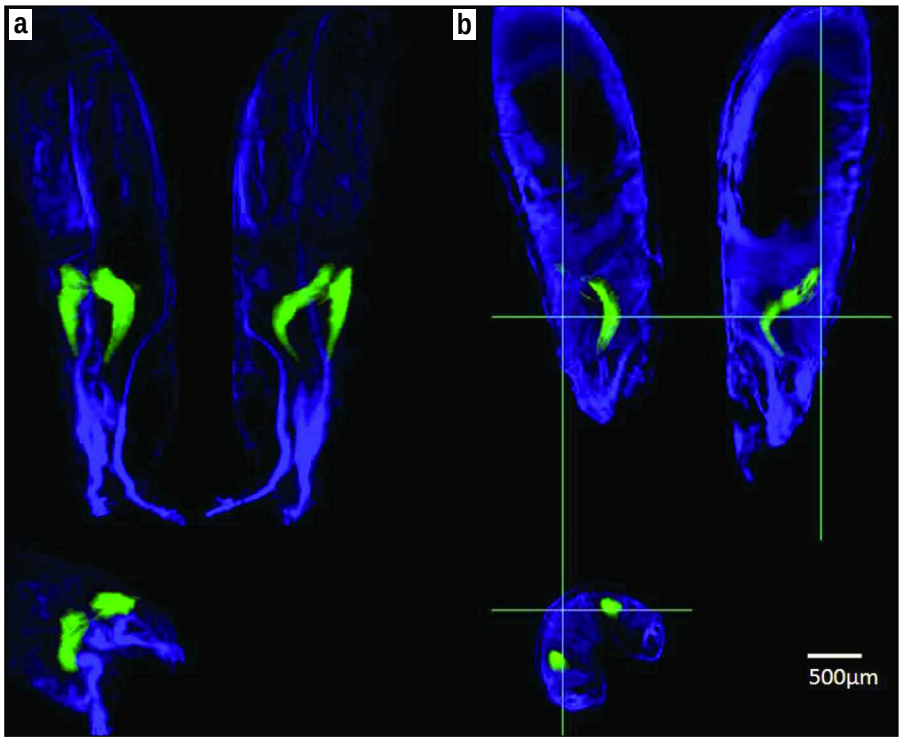

Figure 3:
Overlaid reconstruction of the white light (blue) and fluorescence (green) data of the salivary glands taken in-vivo from an early stage Drosophila melanogaster pupa. (a) 3D overlay. (b) Internal sections longitudinal and transversal through the specimen.

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