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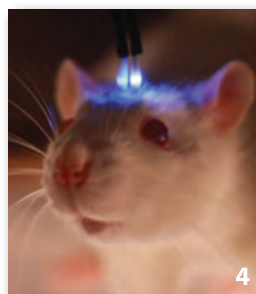

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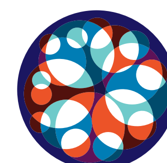

BIOCHEMICAL SOCIETY

Novel 3D imaging platform tracks cancer progression *in vivo*

James McGinty, Paul French (Imperial College London, UK) **and Paul Frankel** (University College London, UK))

Optical imaging underpins biomedical research in many respects and recent decades have seen spectacular advances, particularly in fluorescence imaging where genetic engineering approaches to labelling have been combined with new light sources, detectors and data analysis techniques to provide capabilities like super-resolution beyond the diffraction limit, exquisite spectroscopic contrast for molecular readouts and high-speed image capture for *in vivo* and high-throughput applications. However, the main impact of such advanced instrumentation and data analysis has been to provide unprecedented quantitative 2D and 3D information concerning samples compatible with microscopy where volumes of less than 1 mm^3 are typically imaged in a single 'acquisition'. The ability to view and measure cellular processes and signalling pathways in live cells has been a significant advance for biomedical research and drug discovery. However, for conventional microscope-based assays and experiments, the samples typically comprise thin layers of cells that are not experiencing the same signals that they would in a 3D tissue context and any findings may not directly translate to live organisms. It is desirable to study disease processes in live intact organisms that can provide appropriate physiological complexity. For cancer studies, recent research from our group shows that optical tomography can be used to directly monitor *in vivo* changes in tumour growth and vascular development in a zebrafish cancer model over time. This technique not only improves the value of the collected data, but if used on a wider scale should result in a reduction in the number of animals used in biomedical research.

The rise of fluorescent proteins

The development of target-specific labelling strategies – particularly the ability to express genetically encoded fluorescent proteins in live cells[1] – has enabled cellular processes and signalling pathways to be visualized and quantified. For convenience, such studies are usually undertaken in thin cell cultures (typically on microscope coverslips), but there is an increasing appreciation that the behaviours observed in 2D cell mono-cultures cannot necessarily be directly translated into an *in vivo* context[2]. This is particularly important for understanding disease processes and determining the efficacy, safety and off-target effects of therapies in the drug discovery pipeline. Subsequently, there has been a drive to develop imaging techniques and assays to study disease mechanisms in more realistic physiological contexts. Ideally, preclinical studies should be undertaken in disease models that are as close to humans as possible. However, this aspiration is set against the ability to genetically manipulate the organisms and considerations of accessibility for optical and other readouts.

Murine models are widely used because of their genetic tractability and physiological similarity to humans but they are not optically accessible and the gold standard for preclinical readouts of disease remains histopathology, where the animal is sacrificed and tissue sections produced from regions of interest are stained and imaged at high resolution using optical microscopy. Unfortunately, this can only be performed at a single timepoint per animal and, because only a finite number of sections can be produced, the volumetric sampling may miss important features in heterogeneous tissue. Furthermore, the whole process is time-consuming, requiring significant manual processing and the small fields of view typical of microscopy must be stitched together to map significant fractions of the animal. Fluorescence microscopy can be implemented directly *in vivo* but the limited field of view and the strong absorption and optical scattering experienced by light in tissue limits the range of physiological contexts that can be accessed. Furthermore, the process usually involves invasive procedures with animals that must be euthanized. Currently therefore, whole-animal preclinical imaging in mammals mainly relies on modalities like X-ray computed tomography (CT), magnetic resonance imaging and positron emission tomography, which cannot realize the high (cellular) resolution or molecular contrast and specificity that are available with fluorescence imaging. Fluorescence imaging in intact mice can be

realized using fluorescence molecular tomography and similar techniques[3] that essentially consider the statistical properties of light transport in biological tissue and usually rely on simplifying assumptions to analyse signals based on scattered photons, such as the diffusion approximation. These approaches can reconstruct maps of the tissue's optical properties using inverse scattering techniques, but produce images with spatial resolution limited to greater than 1 mm in mice – significantly degraded compared with the optical diffraction limit.

Potential of *in vivo* optical imaging

Higher resolution *in vivo* optical imaging is possible in smaller transparent organisms such as the nematode worm (*Caenorhabditis elegans*), fruit fly (*Drosophila melanogaster*) and larvae of fish such as the zebrafish embryo (*Danio rerio*), that typically entail imaging through a path of less than 1 mm and for which the usual range of genetic tools are available. This regime is described as 'mesoscopic' imaging and is a very active field that encompasses techniques such as optical projection tomography (OPT)[4] and light sheet microscopy[5], which can provide high-speed, high-resolution 3D imaging for volumes up to ~1 mm in size. While nematodes and flies are non-vertebrates, zebrafish physiology is closer to that of humans and zebrafish larvae are finding increasing interest as a convenient disease model for biomedical research and drug discovery[6]. However, limiting the application of zebrafish studies to embryos also limits the research opportunities, as the larvae are physiologically immature and do not possess fully developed body systems, such as vasculature and immune systems. In this respect, studies using adult zebrafish models are more desirable. However, adult zebrafish reach up to ~1 cm in diameter and ~5 cm in length and so are too large for whole-body imaging in a microscope. Furthermore, whereas zebrafish larvae can be kept optically clear, wild-type zebrafish are pigmented – resulting in significant optical scattering and absorption.

The use of zebrafish as *in vivo* models to study cancer is increasing as they possess various advantages over their mouse counterparts such as easier genetic manipulation and a broader range of imaging opportunities with transparent lines. In relation to translational research, the histological appearance and gene expression profiles of tumours have been shown to be highly conserved between humans and zebrafish[7]. Such histological and genetic similarities suggest that pathogenesis is similar between these species, thus validating the use of these organisms as faithful cancer models.

We have developed a whole-animal 3D optical imaging platform that takes advantage of non-pigmented zebrafish mutants, of which the adults are sufficiently transparent to permit optical readouts[8,9,10]. This platform is based on OPT of adult fish that are immobilized under anaesthetic and utilizes a compressive sensing approach[10] to minimize the data acquisition time and therefore enables the fish to be recovered and reimaged over an extended time course for longitudinal studies[11].

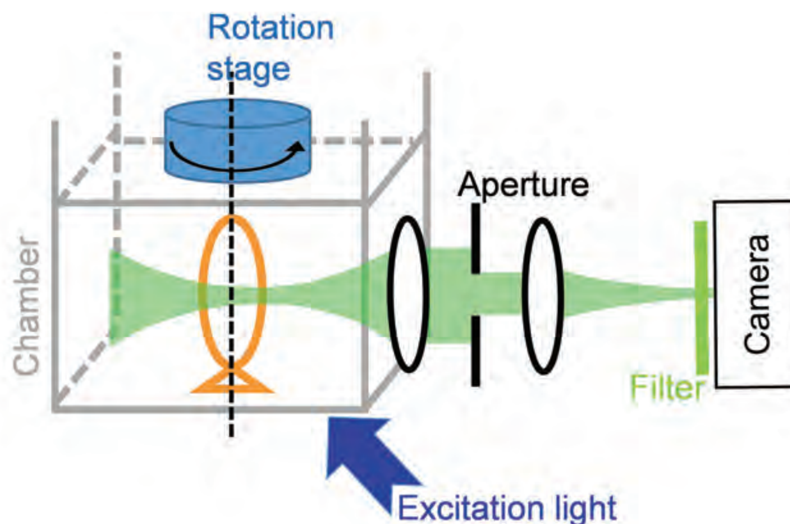

Figure 1. Schematic of an OPT system.

Optical projection tomography of live zebrafish

OPT can be described as the optical equivalent of X-ray CT. It entails rotating the sample and acquiring a series of wide-field fluorescence images at a number of different angular projections, as illustrated in Figure 1. The sample is suspended from a rotation stage in a chamber of refractive index matching fluid (for *in vivo* imaging this is just water). Appropriate excitation light illuminates the sample (in wide-field) to generate fluorescence, which is imaged onto a camera using an imaging system with an aperture. The radius of this aperture is set such that the front half of the sample is imaged 'in focus', providing a projection image. The sample is then sequentially rotated and imaged at a range of angles until it has stepped through a full 360° rotation.

Following the standard formalism of CT[12], each pixel in the wide-field fluorescence image can be considered as the sum of the fluorescence signal along a 'line-of-sight' perpendicular to the camera sensor – or in other words, the 2D wide-field image is a 'projection' of the 3D fluorescent volume. This is analogous to an X-ray image containing information about the absorption of a 3D sample along 'lines-of-sight' and the acquisition and reconstruction procedure are equivalent to X-ray CT: the 3D fluorescence image is reconstructed from the set of angular projection images using filtered backprojection (FBP). An important assumption for FBP, as in X-ray CT, is that the detected light has travelled in straight lines from the sample to the detector,

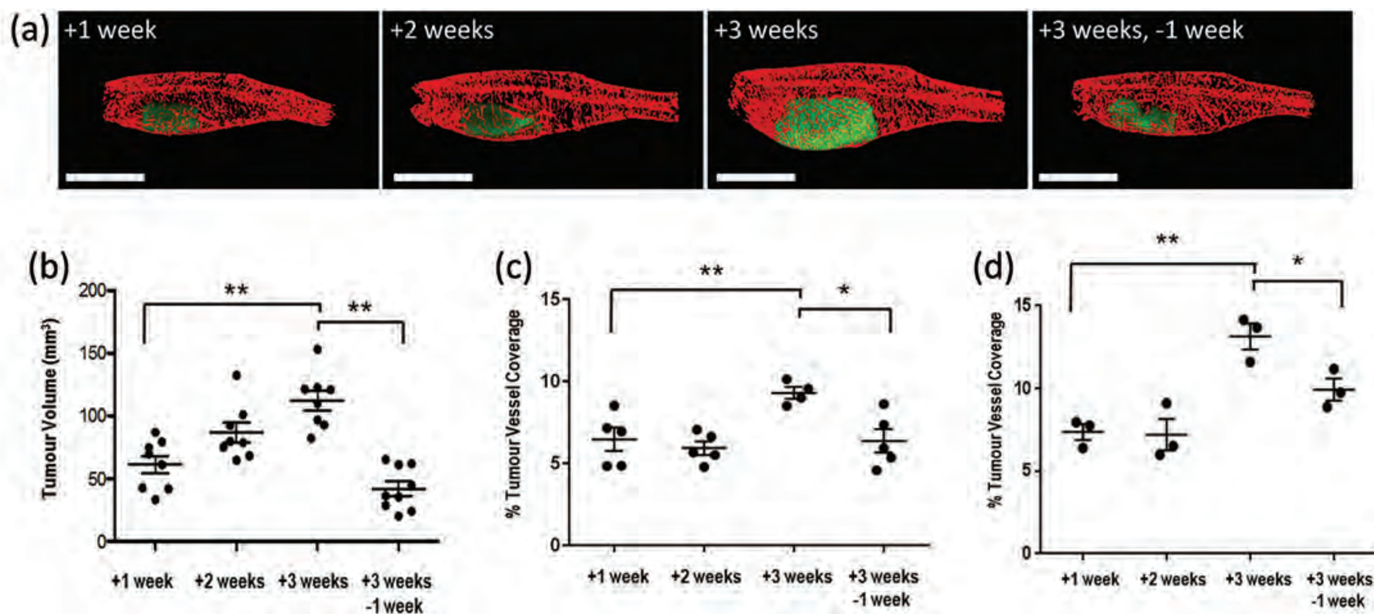

Figure 2. (a) segmented reconstruction of *in vivo* OPT data with tumour (green) and vasculature (red), and plots showing **(b)** tumour volume and **(c)** % tumour vascularization determined from the *in vivo* OPT data and **(d)** measurements based on *ex vivo* immunohistochemistry. Each point is an individual fish. Scale bar 5 mm.

therefore the light should not have been scattered and the sample being imaged should lie within the depth of field of the imaging lens. The requirement for the sample to be non-scattering means that OPT has typically been used to image 'chemically cleared' samples[4], for which water in the sample has been replaced by a liquid of higher refractive index in order to minimize the refractive index variation between different tissue components and therefore to minimize optical scattering. Since this chemical treatment can only be performed *ex vivo* on fixed samples, it is necessary to realize *in vivo* OPT with transparent organisms. As discussed above, we have established that non-pigmented adult zebrafish mutations are sufficiently transparent to enable OPT to be performed *in vivo* on anaesthetized samples.

Imaging tumour growth and vascularization

Figure 2 illustrates how whole-body OPT of a zebrafish cancer model can enable tumour growth and vasculature to be studied. We imaged 'transparent' zebrafish in which the growth of a liver tumour expressing green fluorescence protein could be prompted by exposure to a chemical inducer[10]. This disease model also expresses mCherry fluorescence protein in the endothelial cells of the blood vessels. By mapping the 3D distribution of green and red fluorescence in these fish through *in vivo* OPT, we could measure tumour and vasculature development in a minimally invasive way, requiring less than 10 minutes to acquire the data in both red and green fluorescence channels. To acquire the image data in such a short time we applied a compressed sensing approach where we acquired only 64 angular projections for each OPT data set (rather than the ~600 projections required for lossless reconstruction using standard FBP) and reconstructed the images iteratively as described in [10].

To validate our platform, we performed a cross-sectional study of tumour progression over 3 weeks followed by 1 week without inducer, which results in tumour regression. Typical segmented reconstructed tomographic images acquired over this time course of tumour (green) and vasculature (red) are shown in Figure 2(a). Tumour and segmented vasculature 3D images were then analysed in terms of tumour volume and vessel properties (e.g. branching, average vessel length, tortuosity, etc.[11]). Figure 2(b,c) show quantitative measurements of tumour progression and vascularization derived from the 3D reconstructions of the *in vivo* OPT acquired data. Figure 2(d) shows the corresponding changes in vascularization measured using immunohistochemistry. Importantly, the similarities observed in the comparative analysis of OPT vs immunohistochemistry of the tumour vasculature validates our approach.

Since OPT data acquisition is non-invasive, it does not require the zebrafish to be sacrificed and so repeated measurements can be undertaken for longitudinal studies. Importantly, the OPT data is whole-body and not limited to discrete spatial sampling, unlike histopathology. This means that OPT could potentially readout non-local effects/structures like metastasis, which would require significantly more pathological analysis and/or would otherwise be missed.

Future directions

This initial study demonstrated that *in vivo* OPT can be used to monitor vascular changes associated with tumour growth/recession in live adult zebrafish with similar quantitative readouts to those obtained from histopathological assessment. Unlike histopathology, however, OPT interrogates the whole zebrafish and permits longitudinal studies. This will lead to improved data consistency by reducing the impact of biological variability between different fish. In turn, this can lead to a reduction in the total number of zebrafish required to produce statistically significant readouts for assays of cancer progression and the response to potential therapies.

Our study[11] was limited to fluorescence intensity imaging but more sophisticated fluorescence imaging techniques, such as spectrally and lifetime-resolved fluorescence imaging applied in microscopy, can also be implemented with OPT. For example, we have demonstrated that Fluorescence Lifetime Imaging Microscopy (FLIM) OPT can be applied to provide 3D quantitative readouts of genetically expressed Förster resonance energy transfer (FRET) biosensors, specifically mapping radiation-induced apoptosis in zebrafish embryos using FLIM OPT of a FRET biosensor for caspase 3[13].

We believe that this work illustrates how the combination of semi-transparent model organisms that can be genetically manipulated with whole-body 3D imaging techniques can be used for both fundamental biology and drug discovery and efficacy studies. It can take advantage of the significant developments made in fluorescent reporters developed for cellular assays, transferring them to *in vivo* assays, including longitudinal studies with the potential to improve data consistency and reduce the numbers of animals required for biomedical research and drug discovery. ■

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James McGinty is a Senior Lecturer in the Department of Physics at Imperial College London. His initial research career concentrated on developing instrumentation and analysis software for time-resolved fluorescence imaging with particular emphasis on fast acquisition rates. His current research concentrates on translating and applying quantitative microscopy techniques to more challenging and/or physiologically relevant 3D samples, including cm-sized resected tissue volumes, zebrafish and mice. Maintaining the same optical contrast mechanism across the imaging scales should lead to improved correlation between initial in vitro *cell and subsequent* in vivo *measurements.*

Paul French is Professor of Physics and former Head of the Photonics Group at Imperial College London. He has also worked at the University of New Mexico and AT&T Bell Laboratories. His research has evolved from ultrafast dye and solid-state laser physics to biomedical optics with a particular emphasis on FLIM for applications in molecular cell biology, drug discovery and clinical diagnosis. His current portfolio includes the development and application of multidimensional fluorescence imaging technology for microscopy, endoscopy and tomography.

Dr Paul Frankel is a Group Leader in the Division of Medicine at University College London and a consultant for Magnus Life Sciences. His research specialises in the development of novel molecular targeted agents for the treatment of cancer. Dr Frankel received a PhD in Molecular Biology from the City University of New York Hunter College, specialising in cancer cell signalling. He then moved to the UK to undertake post-doctoral studies in the Laboratory of Professor Chris Marshall FRS at The Institute of Cance Research. Dr Frankel's group combine molecular analysis of cell signalling required for cancer cell motility with state-of-the-art 3D imaging technologies and are working on multiple drug discovery activities.

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