OPTOACOUSTIC IMAGING

By Daniel Razansky, Claudio Vinegoni, and Vasilis Ntziachristos

Unprecedented at the

New optical and optoacoustic methods are solving the bioscientist's conundrum of imaging deep into living tissues at high resolution.

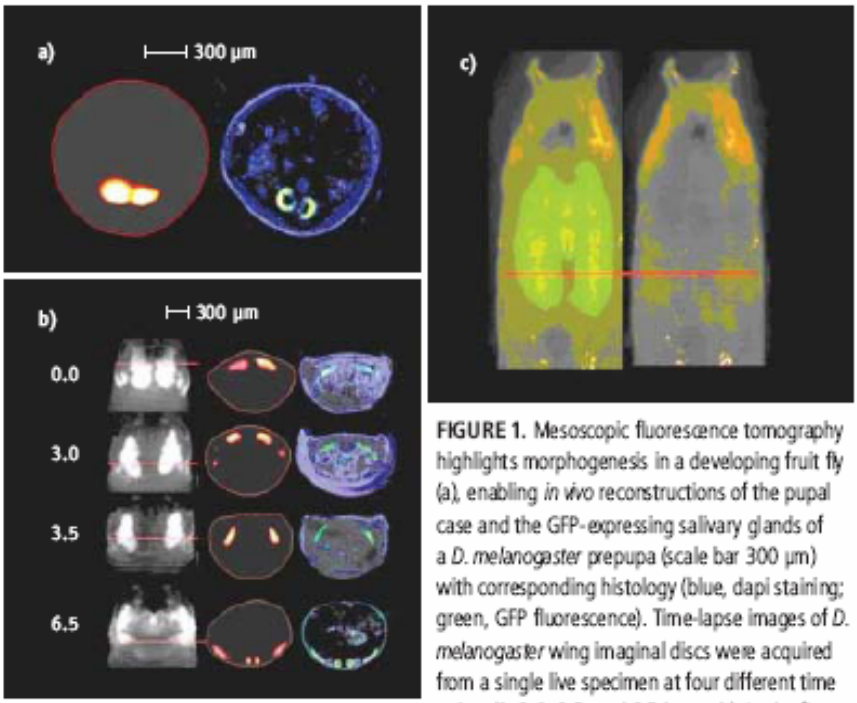

FIGURE 1. Mesoscopic fluorescence tomography highlights morphogenesis in a developing fruit fly (a), enabling *in vivo* reconstructions of the pupal case and the GFP-expressing salivary glands of a *D. melanogaster* prepupa (scale bar 300 µm) with corresponding histology (blue, dapi staining; green, GFP fluorescence). Time-lapse images of *D. melanogaster* wing imaginal discs were acquired from a single live specimen at four different time points (0, 3.0, 3.5, and 6.5 hours; b). In the first column one projection at 0 degrees with respect to the pupa's dorsal view is visible. In the second column the reconstructions correspond to the sections indicated by the red lines. The third column demonstrates comparison with histology and proves good correlation. Planar imaging produced another view of the Drosophila's pupal case and GFP-expressing salivary glands (c).

Progress in the biological sciences has been tied largely to 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. This modality has enabled such key work as visualization of complex molecular pathways and observations of morphological development.

Unfortunately, though, the limits of optical imaging have restricted the study at the organ and organism level with researchers relying mainly on dead-specimen imaging because histology or immunohistochemistry on thin sections yields minimal photon scattering. The need to study evolution, function, and disease in unperturbed environments over time has tasked modern optical visualization with the challenge of *in vivo* application.

Because of severe light scattering (diffusion) in tissues, however, living organisms are largely inaccessible by current optical imaging methods beyond a depth of a few hundred microns. Therefore imaging of many important model organisms and organs has been limited to transparent (e.g., embryonic) stages of development. This makes it highly challenging to relate embryonic cellular and molecular mechanisms to consequences in organ function and animal behavior in more advanced stages. (See "What if there were a genie in your microscope?" www.biooptics world.com/articles/329479.)

Our international group of researchers is developing imaging approaches and technologies to enable high-resolution visualization of tissues and biomarkers at greater depths.

Micro, macro, meso

The underlying physical barrier for extending high-resolution optical imaging beyond several hundred microns is light diffusion. Photons interact with cellular interfaces and organ-

in vivo views mesoscopic scale

elles leading to multiple scattering within the specimen.[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 typically limited to a depth of 0.5 mm in living tissues. (See "Success with advanced microscopy," www.bioopticsworld.com/webcastDetails.html?id=915) And recent efforts to limit scattering in entire embryos require special chemical treatment of the specimen—which necessitates post-mortem observation.[2,3]

Macroscopic optical imaging has recently evolved as an alternative for visualizing large diffuse specimens. It uses fully diffusive photons, typically from structures that are larger than 1 cm. An advanced form, fluorescence molecular tomography (FMT) illuminates the sample under investigation at multiple projections and uses mathematical models—combined with the actual capturing of photon propagation through tissue—to reconstruct the underlying imaging contrast. Compared to microscopic three-dimensional "tissue-sectioning" imaging, tomography, and reconstruction 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 implementations have proved able to three-dimensionally image biodistribution of fluorochromes in entire animals, and molecular pathways of cancer and cardiovascular disease, for example. Optical tomography, when applied in diffusive tissues with dimensions larger than 1 cm, can produce low (approximately 1 mm) spatial resolution.

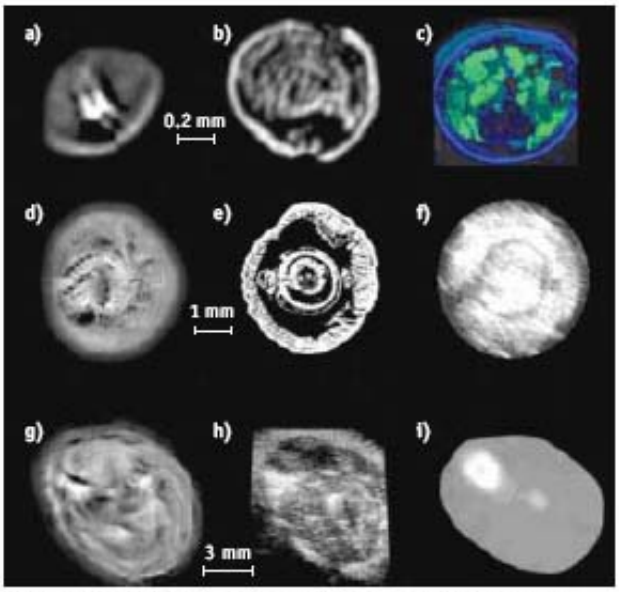

FIGURE 2. Selective-plane optoacoustic tomography (SPOT) enables imaging of of mesoscopic-scale objects such as an intact *D. melanogaster* pupae, including its darkly colored (highly absorbing) sensory organ(a); and its salivary gland area (b). Histological section of the pupa at the salivary gland area (blue indicates DAPI staining; green shows GFP in the fatty structures) corresponds well with the SPOT imagery (c). Similarly, a SPOT image of *lumbricus terrestris* (earthworm; d) corresponds with an anatomical diagram (e) and an image acquired using high-resolution ultrasound system at 25MHz (f). Finally, an optoacoustic tomography reconstruction image obtained from the pelvic limb of a wild-type Balb/c mouse (g), and the corresponding ultrasound (h), and micro-CT (i) images.

Mesoscopic fluorescence tomography

For organisms and tissues at the mesoscopic scale—with dimensions between 1 mm and 1 cm—neither ballistic nor diffuse photon-propagation regimes apply. For this reason, real-time whole-body *in vivo* imaging via optical microscopy (below 1 mm) and macroscopy (above 1 cm) have been inadequate to follow the dynamics and coordination of development in developing insects, animal embryos, or small-ani-

DANIEL RAZANSKY *is deputy director of the Institute for Biological and Medical Imaging (IBMI) at the Technical University of Munich and Helmholtz Center Munich, where he also heads the Laboratory for Experimental Biological Imaging Systems (Munich, Germany).* CLAUDIO VINEGONI *directs the In Vivo Microscopy Core at the Center for Systems Biology at the Massachusetts General Hospital and Harvard Medical School (Boston, MA, USA).* VASILIS NTZIACHRISTOS *is the director of IBMI and chair for Biological Imaging at the Technical University of Munich. Contact Dr. Razansky at dr@tum.de.*

mal extremities.

Our group recently demonstrated that with the help of a photon transport description using a modified Fermi simplification to the Fokker-Planck approximation, fluorescence tomography can image three-dimensional structures of developing Drosophila *in vivo* and over time.[4] In particular, we followed the morphogenesis of GFP-expressing salivary glands and wing imaginal discs in the opaque Drosophila pupae in real time (see Fig. 1). Our mesoscopic fluorescence tomography (MFT) technique is based on a modified standard laboratory microscope. While the spatial resolution of the approach is exchanged for penetration depth, the method adds a dimension—time—to the study of morphological changes during metamorphosis and potentially as a response to mutations and internal or external stimuli.

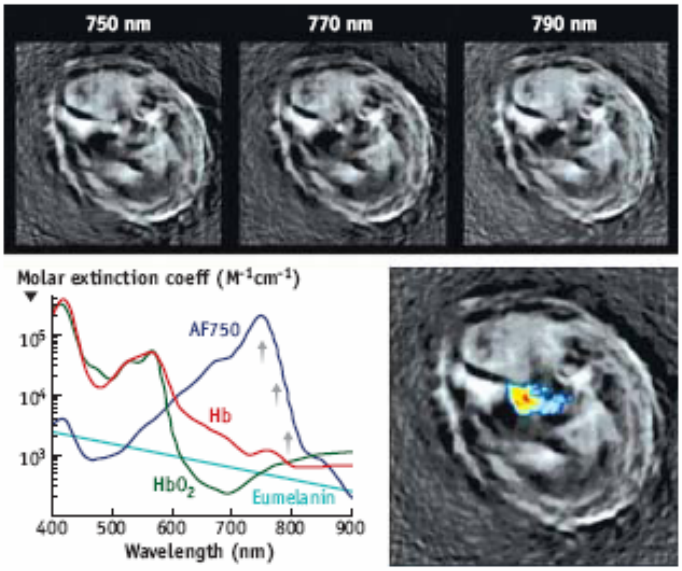

FIGURE 3. Multispectral optoacoustic tomography (MSOT) visualizes distribution of a fluorescent molecular probe (AlexaFluor 750) in a mouse leg: cross sectional reconstructions were acquired at 750, 770, and 790 nm (top row). Absorption is measured as a function of wavelength for AF750 fluorescent probe and is compared with some intrinsic tissue chromophores (lower left); arrows indicate the three wavelengths used to spectrally resolve the probe location. A corresponding spectrally resolved MSOT image incorporates measurements at all the three indicated wavelengths (in color), superimposed onto a single-wavelength anatomical image (lower right).

Selective-plane optoacoustic tomography

Optoacoustic (or photoacoustic) tomography is a hybrid imaging modality that has demonstrated unprecedented high-resolution imaging of chromophore distribution and vasculature deep in tissues of small animals.[5] When applying pure optical imaging to diffuse tissues, the spatial resolution is always exchanged for penetration. Therefore, as the size of the imaged object grows, imaging resolution diminishes. It is even possible to perform optical tomography through entire mice with high sensitivity—but at low resolution of about 1 mm or less.[6]

By contrast, optoacoustic interrogation combines high optical absorption with good spatial resolution, because the approach includes ultrasonic signals induced by absorption of pulsed light. The amplitude of the generated broadband ultrasound waves reflects local optical absorption properties of tissue.

Our selective-plane illumination optoacoustic tomography technique renders high-resolution whole-body visualization of intact mesoscopic-scale optically diffusive organisms.[7] While optoacoustic imaging of tissues typically target endogenous tissue contrast, primarily resolving oxy- and deoxyhemoglobin and different vascular structures, the new method provides good contrast from other tissues such as fat, bone, and other internal structures. The method has proved useful for visualizing several optically diffusive model organisms (see Fig. 2). Its resolution is constant for the entire penetration range of several millimeters to a centimeter of tissue, and is limited only by the useful bandwidth of the ultrasonic detector, which can be further improved over time to attain better performance. With advances in detection technology, it offers a platform for mesoscopic imaging with resolution that can practically approach 20 µm or less.

Multispectral optoacoustic tomography (MSOT)

In addition to rich intrinsic tissue contrast, optoacoustic imaging can be used to visualize exogenous molecular and functional markers. This is done by using pulsed illumination at multiple wavelengths so that distinct spectral signatures from certain biomarkers can be resolved over background tissue absorption by applying spectral processing. In this way, additional information contained in the optical spectrum—such as fluorogenic or chromogenic biomarkers associated with gene expression, morphogenesis, or disease progression—can potentially be resolved.

Recently developed multispectral optoacoustic tomography (MSOT) enables high-resolution 3-D visualization of molecular probes located deep in scattering living tissues.[8] It can simultaneously deliver anatomical, functional, and molecular information with high resolution and deep penetration with femtomole-level sensitivity (see Fig. 3).

Bringing the best out of the spectrum

The ability to optically interrogate and visualize intact organisms beyond the miscroscopy limits is important, for instance, for accelerating the study of functional gemomics and proteomics. Our work focuses on the development of biological imaging methods capable of visualizing various molecular biomarkers, such as chromophoric or fluorescent dyes and proteins, deep in diffuse organisms *in vivo*. These methods can enable a new generation of biological imaging by providing molecular and functional information simultaneously with the necessary anatomical-reference images.

A particular strength is the ability to scale with different organism and tissue sizes from submillimeter to 1 cm and beyond. Indeed because many relevant biological samples and model organisms lie in this range, these techniques are valid tools for imaging organisms such as worms, insects, and vertebrates such fish and small mammals—as well as these animals' extremities.

These methods fill a significant area within the biological imaging arena. They may not only significantly enhance the usefulness of current transgenic lines, but may call for the development of novel reporters, for example, to indicate progressing obesity, arthritis, plaque in the nervous system, or atherosclerosis. «

REFERENCES

1. V. Ntziachristos et al., *Nature Medicine 8*, p. 757 (2002).
2. J. Sharpe et al. *Science 296*, p. 541 (2002).
3. H.U. Dodt et al. *Nature Methods 4*, p. 331 (2007).
4. C. Vinegoni et al., *Nature Methods 5*, p. 45 (2008).
5. X. Wang et al., *Nat. Biotechnol. 21*, p. 803 (2003).
6. V. Ntziachristos, et al.. *Nat. Biotechnol. 23*, p. 313 (2005).
7. D. Razansky et al., *Phys. Med. Biol. 54* (2009).
8. D. Razansky et al., *Optics Letters 32*, p. 2891 (2007).

pioneers *cont.*

got all six grants the first time I applied for them. I just had to say, 'this is one of my first proposals in academia.'" He quipped that his grants are sometimes turned down now.

He was chosen, in 2004, as one of nine winners of the inaugural NIH Director's Pioneer Award, which netted a $500,000 discretionary fund each year for five years. This grant allowed the Xie research group to pursue its gene expression and CARS/SRS work, which would have been impossible through conventional funding.

With his Pioneer grant ending this year, funding is an ongoing challenge. However, Xie says that the NIH Pioneer award "has changed the way I do science," making him focus on the big picture. "Of course, the big picture isn't enough, I need to deliver! Fortunately, we did it this time. Now I continually think of exploratory work that has a potential of high return, with the inevitable high risk."

He explains the evolution of his research. "I used to write papers in highly specialized fields that only a few people in the world would read," he says. "When I started to do single-molecule spectroscopy, I found more people cared about our work. After we applied it to enzymology, and now having done single-molecule live-cell studies and SRS imaging, even more people are interested in our work. Gradually, I see our work begins to have an impact. This brings a lot of satisfaction to my students, to my research group, and to myself."

And by the way, he says, "we've got this new idea. If it works, it will be transforming." «