As Clear as a Cell: Researchers See Deeper into Tissue than Ever Before

Brendan Brinkman
Olympus America Inc., 3500 Corporate Parkway, PO Box 610, Center Valley, PA 18034
Brendan.Brinkman@Olympus.com

Introduction

Researchers have long sought to visualize neural projections and other features of animal brains by imaging them using light microscopes. Connectomics studies strive to elucidate the connections among nerve cells, but brain is dense, and gray matter is tremendously light-scattering. So scientists have not been able to peer as deeply into brain tissue as is necessary to view its complex connections and structures without cutting brain tissue into thin sections. The slicing causes damage that makes it even more difficult to reconstruct exactly how nervous system structures are interconnected. Issues with light scattering have also limited studies of microvasculature, developmental structures, and other features in mouse embryos and other tissues. Traditional methods of retrograde or anterograde labeling have been able to provide high specificity of connection circuitry, but at the price of losing the organizational context of the network. In addition, there is a limit to the practical number of labels that can be used at once. Even multicolor methods have required serial section reconstruction.

Until recently, to image even a millimeter deep into living or fixed tissue would have been unthinkable. The development of multiphoton microscopy, first patented by the laboratory of Watt Webb of Cornell University in 1991 [1], was a major breakthrough. Multiphoton microscopy involves the excitation of molecules by the simultaneous absorption of two or more photons that combine to provide stimulation of fluorophores. Multiphoton systems rely on rapidly pulsed lasers to coordinate the timing of photon delivery directly to the focal plane, reducing average power to the sample while maintaining high peak power to generate fluorescence excitation. Out-of-focus light coming from the sample is eliminated. By combining two or more photons of relatively long wavelengths (that is, lower energy) to achieve excitation, imaging can be achieved at much deeper levels within tissue because of the lower scattering of infrared light. The advent of commercial multiphoton systems has allowed images to be captured as deep as 700 microns or more into tissue.

Methods and Materials

New technology has been developed that significantly reduces light scattering and allows imaging of fluorescent markers several orders of magnitude farther into fixed specimens, up to 8 mm deep even within very dense brain tissue. The technology comprises a complete multiphoton system incorporating a clearing reagent, first developed by Dr. Atsushi Miyawaki and his colleagues at the RIKEN Brain Science Institute in Japan, and two new 25× objectives developed by Olympus Corporation specifically for use with the reagent.

The reagent was first described in August 2011 [2] and has since been described in news media throughout the world. It literally makes tissue transparent. Even more important, the reagent avoids fluorescence quenching—a decrease in the intensity of signals emitted by fluorescent markers within tissue. The reagent, originally called Scale, may be made inexpensively with commonly attainable materials or purchased commercially (under the name SCALEVIEW-A2) from Olympus.

Optimizing imaging performance using the new reagent required new optics. Dr. Miyawaki's team turned to Olympus Corporation three years ago for support in developing microscope objectives that would maximize performance. The primary challenge was to create objectives with long working distances for focusing through a significant depth of material, while also maintaining the resolution needed to make imaging at that depth useful. The industry-research partnership resulted in development of two 25× long-working-distance objectives engineered to match the 1.38 refractive index of the new reagent.

The lenses were specially designed with correction collars that allow for optimization of lens focus within tissue. Correction collars move lenses within an objective, allowing for correction of spherical aberration that occurs because of refractive index mismatch between immersion media and the sample; the lenses used were designed with correction collars capable of matching the clearing solution, which was also used as an immersion medium. Unlike many correction collars, which change lens focus position when adjusted, or do not offer correction throughout the entire working distance of the lens, the SCALEVIEW objectives offer an extensive range of adjustment capability throughout the working distance of the lens, while minimizing any change to lens focus position. This is important because, without being able to correct for the depth of the tissue, sharp images cannot be achieved throughout the entire depth of the specimen, even if a lens has a long working distance. Thus, correction collar designs that produce large changes in focus when properly adjusted for the depth of imaging can make it difficult to adjust during experimentation (Figure 1).

The first of the two objectives was a high-performance 4 mm-working-distance objective with a numerical aperture (NA) of 1.0, which has enabled researchers to produce vivid 3D images of structures deep inside mouse brains and other animal organs. More recently, the company introduced an 8 mm-working-distance objective with an NA of 0.9. By combining extra-long working distances with unusually high NAs, these objectives are able to deliver images with outstanding clarity and detail at unprecedented depths.

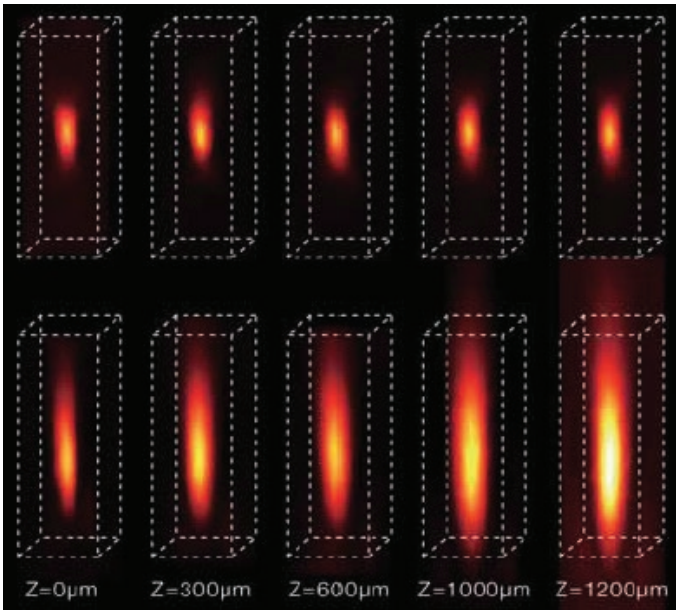

Figure 1: Comparison of axial resolution due to refractive index spherical aberration correction in lens with correction collar set (top, Olympus XLPLN25XWMP), and without any correction collar (bottom, Olympus XLUMPLFL20XW). Images of 0.5 μm fluorescent microspheres produced by the Olympus Fluoview FV1000MPE system.

Both objectives have been optimized for use with the Olympus FluoView FV1000®MPE and the new FV1200MPE multiphoton systems. Although the new lenses can be used for visible wavelength excitation, the concept behind them is to image as deeply as possible with bright, clear, high resolution. In keeping with this goal, Olympus designed the optical coatings to provide the greatest transmission when using multiphoton excitation, allowing superior imaging at depth. By comparison, some other long-working-distance objectives have been optimized for UV-wavelength work, making them less useful for the deep imaging that is characteristically done with multiphoton microscopy.

Results

Using the new system, scientists have captured high-resolution 4 mm-deep images in mouse that clearly show structures extending from the brain surface to the hippocampus, a crucial area for memory function. For some of these images (Figure 2), automation and software control have driven 3D acquisition of nearly 9,000 images that have been stitched together to generate the data set. Final images rendered in 3D visualization programs trace the contours of the neuronal connections. Images that extend to 8 mm are also possible, opening up the potential of visualizing almost through the entire depth of a mouse brain.

According to a statement by RIKEN [3], Dr. Miyawaki and his team have already used the system to study neurons in the mouse brain at an unprecedented depth and level of resolution, shedding light on the intricate networks of the cerebral cortex, hippocampus, and white matter. In initial experiments the team visualized the axons connecting the left and right hemispheres and blood vessels in the postnatal hippocampus in greater detail than ever before [2].

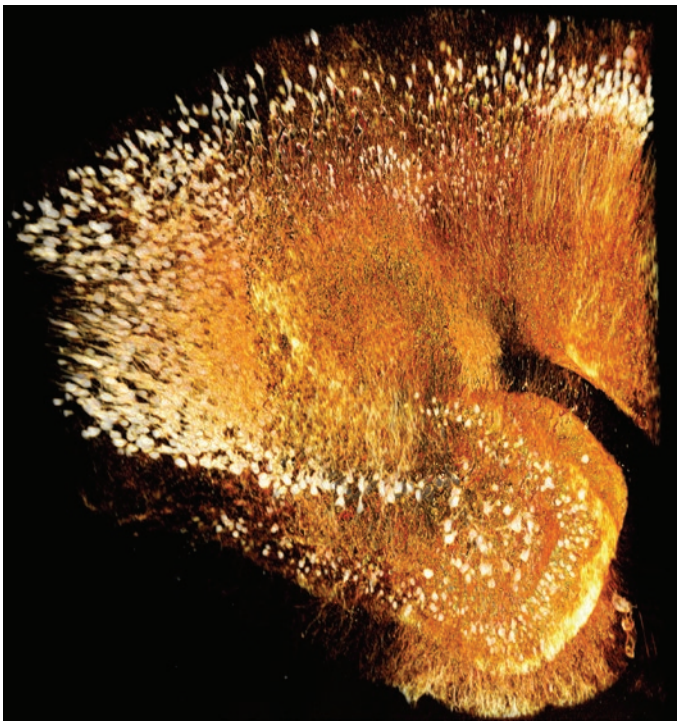

Figure 2: Mouse brain showing fine filamentous structures of the hippocampus. The tissue was rendered optically transparent using a new technique developed by Dr. Atsushi Miyawaki of the RIKEN Brain Science Institute in Japan. The image was obtained using an Olympus FluoView FV1000MPE multiphoton microscope system with the SCALEVIEW immersion 4 mm, 25×, 1.0 NA MPE objective and SCALEVIEW-A2 optical clearing agent. Raw image data courtesy of Hiroshi Harna, Hiroshi Kurokawa, and Atsushi Miyawaki, RIKEN Brain Science Institute, Laboratory for Cell Function Dynamics, Japan. 3D rendering of raw image data was performed using Imaris software (Bitplane). Image width is 3.3 mm.

While neurobiology is an obvious application for SCALEVIEW, there are many other fields of study in which researchers can benefit from seeing more deeply into tissue. Dr. Claudio Vinegoni of the Weissleder Lab at Harvard's Massachusetts General Hospital has been using the SCALEVIEW reagent and lenses to generate deeper views into a range of tissues including pancreas, epididymis, heart, and kidney. Many of these tissues are notoriously difficult to image because of strong light scattering. Dr. Vinegoni's research on the pancreas has focused on islets, the clusters of beta cells responsible for insulin creation. For instance, a single pancreatic islet was cleared in the reagent and then imaged more than 300 μm below the surface, a significant improvement over traditional confocal or multiphoton imaging (Figure 3). The cross section in Figure 4 shows rich structural information on the distribution of cells within the islet. The vascularization associated with islet cell formation is another important measure in this area; visualizing blood vessel networks is a logical use of the same clearing and imaging techniques used by Dr. Vinegoni, Dr. Miyawaki, and others. Images of the whole pancreas up to 4 mm in size and larger have also been captured using the new technique (Figure 5).

Even without the clearing reagent, some researchers are finding benefits to the use of the new objectives alone. Dr. Rachel Wong of the University of Washington is using the objectives to examine zebrafish. The massive front lens of the

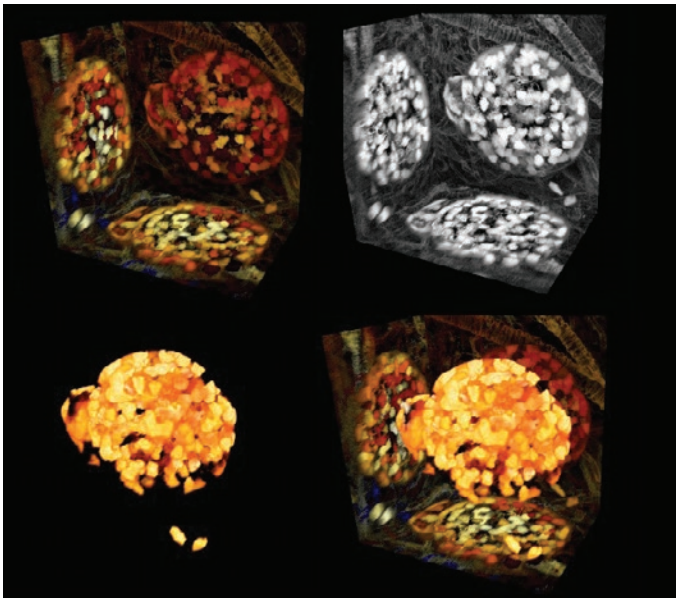

Figure 3: Mouse pancreatic islet cleared with SCALEVIEW-A2 reagent and imaged with 4 mm 25× SCALEVIEW objective. Image of a single pancreatic islet in a transgenic mouse insulin promoter green fluorescent protein (MIP-GFP) mouse. Here the GFP expression is targeted to the beta-cells. The three-dimensionally rendered islet is approximately 100 microns across. Reproduced with permission from Claudio Vinegoni, Mass General Hospital, Boston.

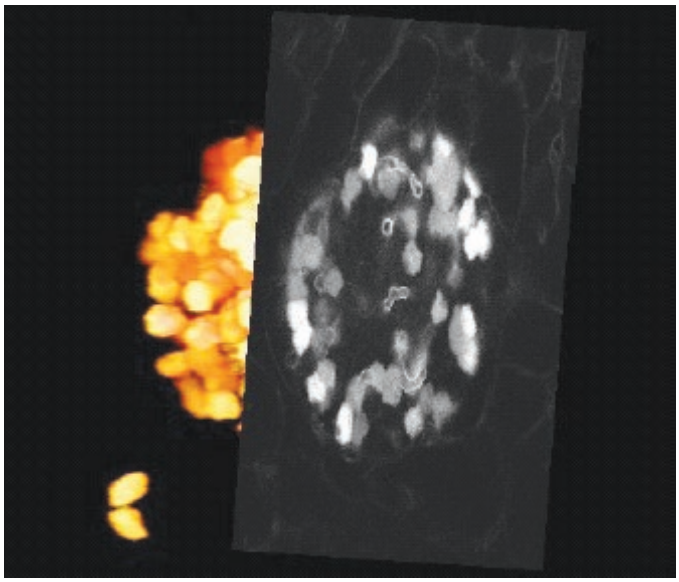

Figure 4: Cross section view of an islet more than 300 μm deep within cleared tissue. The pancreas is typically highly scattering, making it difficult to image individual cells throughout the tissue. After clearing with SCALEVIEW, individual cells can be resolved throughout individual islets deep within the tissue. Cross section reveals the clarity of the specimen throughout the entire islet. Three-dimensionally rendered islet is approximately 100 microns across. Reproduced with permission from Claudio Vinegoni, Mass General Hospital, Boston.

SCALEVIEW objectives is remarkably efficient at collecting light, resulting in strikingly clear images of live, immobilized fish such as the one imaged in Figure 6. Dr. Wong intends to use these lenses in larger animals as well and anticipates that the long working distances will be of use. Other researchers have expressed interest in the extra-long working distances available for patch-clamp recording.

Discussion

Working distance generally has an inverse relationship to the resolving power of a lens, often expressed as its NA. Long-working-distance objectives have traditionally been used primarily in industrial applications and in life science applications, where it has been necessary to image through thick well plates. Only within the last decade have these lenses begun to play a major role in deep multiphoton imaging. Traditional long-working-distance objectives are typically low-magnification and have relatively low NAs; their key utility has been the easy access they provide to specimen regions that are a large distance from the objective. However, life science applications, particularly multiphoton applications, have increased the need for higher NAs, not only for the increased efficiency of photon focusing to create non-linear excitation effects, but also for the increased resolution required to collect meaningful data in tissues and cellular structures. Within this context,

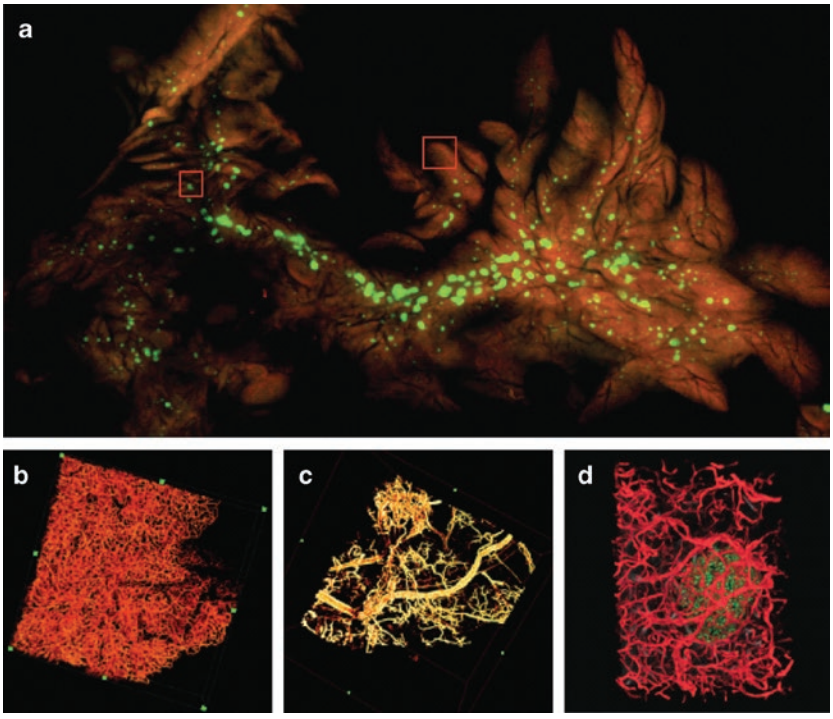

Figure 5: An image of a whole pancreas (a), 4 mm across, in a transgenic mouse insulin promoter green fluorescent protein (MIP-GFP) mouse, with callout areas (indicated by red boxes) shown at (b), (c) and (d). Griffonia simplicifolia-I lectin was injected intravenously to stain the endothelial cells selectively. Green corresponds to GFP expression targeted in the islets' beta cells; red corresponds to the pancreatic vasculature. The larger red box corresponds to the areas of images in (b) and (c). Image (b) shows a large area vasculature overview of the top surface of the pancreas, measuring 1000 μm across. Image (c) shows a higher-magnification view of vasculature measuring 250 μm across. The smaller red box corresponds to image (d). Image (d) shows an islet (green, 100 μm in diameter) nestled within vasculature. Reproduced with permission from Claudio Vinegoni, Mass General Hospital, Boston.

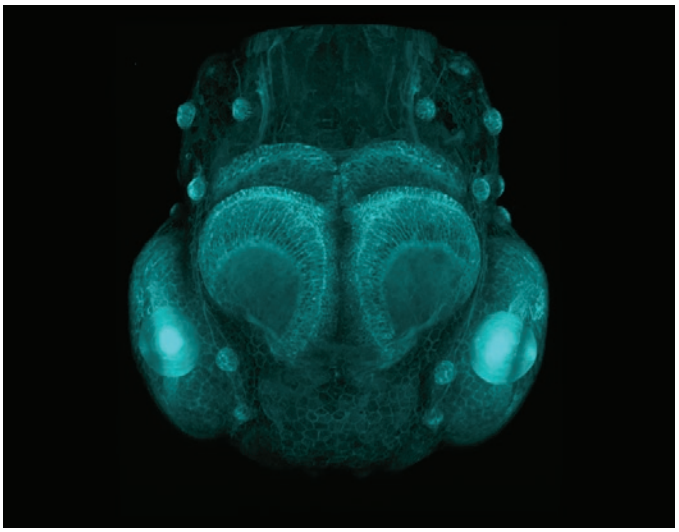

Figure 6: Maximum intensity projection of live zebrafish transgenically expressing yellow fluorescent protein (YFP, here depicted in bluish tones) in a subset of neurons. Zebrafish head is 350 µm wide. Image acquired with 4 mm Olympus SCALEVIEW 25× objective. Reproduced with permission from Rachel Wong, University of Washington.

correction collars in long-working-distance lenses, which had previously been designed to adjust for the thickness of coverslips, slides, or well plates and refractive index changes between immersion media and sample, took on the additional role of adjusting for the thickness of tissue. This has allowed them to correct for light scatter within those tissues and properly focus light to correct for spherical aberration, providing the highest photon flux needed in the focal plane for non-linear fluorescent excitation processes. Dipping objectives allow lenses to use aqueous solutions as immersion media; they have improved NAs while maintaining longer working distances. This is particularly useful for electrophysiology patch clamping, but as multiphoton technology has developed, researchers have required both higher resolution and deeper image penetration. Ultimately this has driven lens designs that couple low magnifications, large fields of view, long working distances, and high NAs with correction collars that adjust for the ever-increasing depths made possible by long-wavelength imaging. With new clearing technologies, this lens evolution has reached a new pinnacle: low-magnification objectives such as the new ones described here have high-enough NAs and correction collars enabling them to resolve structures as deep as 8 mm within fixed tissue, particularly when used with the SCALEVIEW clearing reagent and a properly designed multiphoton scanning system.

Since the publication of the Scale technique by Dr. Miyawaki's group, a host of researchers have been working on various new types of clearing methods. Recently the CLARITY method was published by Dr. Karl Deisseroth's lab [4], showing that increased clearing can be achieved. This technique further enhances the possibilities for deep imaging limited only by the working distance of the objective, particularly in fixed tissue. With the increasing focus on brain mapping projects, both in fixed tissue and, with the recently announced NIH BRAIN project, *in vivo* functional mapping, researchers can look forward to ever-increasing possibilities as both optical technologies and clearing techniques advance.

Microscopy has always faced certain fundamental limitations, and the paradigms in which scientists work have been defined by the tools available to them. As science has moved from reductive paradigms to an explicit understanding that context matters for function, tools that can increase the scale of researchers' questions become increasingly important. Clearly, SCALEVIEW is one of those tools.

Conclusion

The new clearing solution renders previously opaque tissue transparent, dramatically reducing light scatter that otherwise would limit the penetration of light within the sample. The two 25× lenses described here take full advantage imaging in cleared tissue. They have coatings optimized for multiphoton imaging and correction to match the refractive index of the SCALEVIEW-A2 solution specifically, with 4 mm and 8 mm working distances and NAs of 1.0 and 0.9, respectively. The lenses can be used with either water or the new clearing reagent to provide researchers with options for high-resolution and long-working-distance imaging. Multiphoton and other nonlinear microscopy techniques are critically dependent on the properties of the pulse IR laser. Complete microscope systems help correct for group velocity dispersion of the pulsed IR light, beam size, and other critical factors for the excitation laser such as optical coatings; they also provide efficient and highly sensitive collection of the emitted light, all of which are factors in the ultimate success of these techniques. When used as part of a specific, integrated multiphoton system, imaging through the entire working distance of the lenses up to 8 mm is now possible within cleared tissue, allowing researchers to generate contiguous 3D data sets vastly deeper and with higher resolution than was possible before.

References

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