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High-Resolution Ocular Imaging: Combining Advanced Optics and Microtechnology

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Optic Nerve Rescue and Restoration Think Tank Attendees

The annual Optic Nerve Rescue and Restoration Think Tank, sponsored by The Glaucoma Foundation, was entitled “High Resolution Imaging of the Eye: Advanced Optics, Microtechnology and Nanotechnology” and held in New York, NY, September 28-29, 2007.

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Abstract

Recent developments in imaging technologies offer great potential for the assessment of retinal ganglion cell disorders, with particular relevance to glaucoma. In particular, advances in this field have allowed unprecedented in vivo access to the retinal layers, using many different properties of light to differentiate cellular structures. This article is a summary of currently available and investigational advanced, high-resolution imaging technologies and their potential applications to glaucoma. It represents the topics of discussion at the annual Optic Nerve Rescue and Restoration Think Tank, sponsored by The Glaucoma Foundation, entitled “High Resolution Imaging of the Eye: Advanced Optics, Microtechnology and Nanotechnology” and held in New York, New York, September 28-29, 2007.

Introduction

Glaucoma is a leading cause of irreversible blindness worldwide. Loss of vision is caused by progressive retinal ganglion cell and axonal death, resulting in a characteristic pattern of optic nerve head and visual field loss.¹ Because glaucoma is a silent and insidious disease, patients often present late in its course, when significant visual loss has already occurred. Furthermore, the gold standard of diagnosing glaucomatous visual field loss by standard automated perimetry detects disease only after a significant proportion of retinal ganglion cells have already been lost,² corresponding to a 10-year delay in diagnosis.³

Currently, lowering intraocular pressure, the most important known risk factor for glaucomatous damage, is the only available therapy for the treatment of glaucoma.⁴ Unfortunately, patients can continue to lose vision despite apparently successful intraocular pressure control. This has highlighted the need for alternative treatment approaches.⁵ However, the evaluation of potential neuroprotective strategies that do not rely on lowering intraocular pressure requires both better defined clinical end points and earlier and better detection and measures of progression. An “ideal” end point in glaucoma would be one that could directly measure retinal ganglion cell dysfunction and disease.

From an anatomic perspective, new noninvasive imaging technologies^{6,7} to identify early changes in the retinal nerve fiber layer and optic nerve head would be valuable. Reaching this goal requires both advances in the resolution of existing technologies and a better understanding of the changes occurring in the optic nerve, including the temporal ordering of these changes. Recent advances in biomechanical engineering suggest that changes in deformation of the lamina cribrosa may provide a quantifiable measure of early glaucomatous changes.⁸⁻¹²

Similarly, our understanding of changes in glial cell behavior and molecular profiles in the optic nerve head may provide biological markers for early changes in this tissue. From a cellular perspective in the retina, it would be valuable to understand the molecular changes associated with both axonal degeneration and retinal ganglion cell soma loss. For example, defining early morphological changes in the retinal ganglion cell soma, such as changes in cell volume, may be detectable. Alternatively, defining metabolic changes, such as differences in energy metabolism or ion channel conductance, may provide markers that could be monitored as markers of retinal ganglion cell function. It is important to verify further the relative timing of events of retinal ganglion cell dysfunction as they occur in the apoptotic pathway so that we can concentrate on events that precede the irreversible stages of cellular degeneration. Essentially, we need to ask what changes occur in affected retinal ganglion cells that may be reversible, thus allowing for better resolution of the effects of protective strategies?

Recent developments in imaging technologies offer great potential for the assessment of retinal ganglion cell disorders, with particular relevance to glaucoma. In particular, advances in this field have allowed unprecedented in vivo access to the retinal layers, using many different properties of light to differentiate cellular structures. The following is a summary of currently available and investigational advanced, high-resolution imaging technologies and their potential applications to glaucoma.

Optical Coherence Tomography

Optical coherence tomography (OCT) is probably the technology that has seen the greatest recent advances in clinical imaging. It uses a low coherence interferometry-based system to measure changes in reflectivity at very high spatial resolution.¹³ OCT has been increasingly used in ophthalmology since 1991, with more than 67 companies now producing OCT-based products for ophthalmic use. Its role in the assessment of macular disease is well established, and in glaucoma it has been advocated for its potential in measuring thicknesses of the retinal layers, including the retinal nerve fiber layer thickness.¹⁴⁻¹⁹

Three-Dimensional OCT

This conventional method of OCT uses a moving reference arm to obtain axial information, with the limitation being the speed of scanning. Increasing the speed has led to great improvements in three-dimensional (3D) imaging capabilities, where a series of multiple x-y scans are reconstructed in the z plane. This has directed the development of “optical biopsy” or “virtual surgery” of retinal pathology.²⁰⁻²³ Three-dimensional OCT produces detailed and striking images of retinal pathology, as illustrated so convincingly by Choi et al.²³ and

Drexler.²⁴ Cross-sectional en-face views and rendering from a virtual perspective provides the potential of measuring changes in the retinal nerve fiber layer over time, using 3-D OCT. However, this method still cannot be used to visualize individual retinal ganglion cells, and it is recognized that further development is needed to establish the capability of this method in glaucoma.

Ultra-High Resolution OCT

Ultra-high resolution OCT achieves much higher spatial resolution, of the order of 2 to 3 μm , compared to standard OCT.²⁵ Unlike conventional OCT, which uses monochromatic visible light, ultra-high resolution OCT uses a wide-band light source. The use of femtolasers as the light source improves the resolution even more,²⁶⁻²⁹ but the cost of femtolasers currently makes it difficult for them to be used on a wide scale.

Fourier or Spectral Domain OCT

Another modification of OCT is Fourier domain or spectral domain OCT. These machines use a spectroscope to analyze the intensity of each wavelength making up the beam independently. Because this methodology does not rely on the speed of a moving arm for each scan, acquisition of images is much faster. High-definition images of the retina and optic nerve head have been obtained with this technology, with image sizes of up to 10,000 pixels.³⁰⁻³⁴ However, there are limitations because the sensitivity of the technique varies with depth and tissue thickness, and the limit of depth range is currently 1.5 mm. Because this is an emerging technology, new approaches have been used (eg, use of a sweeping laser source that produces 236,000 scans/sec, which is 500 times faster than conventional CT). The power of this technology can be illustrated by the fact that individual red blood cells can be imaged in vivo in a quail's beating heart.³⁵

Adaptive Optics OCT

Adaptive optics was originally developed by astrophysicists to improve visualization of outer space. By correcting for optical aberrations in real time using a deformable mirror, adaptive optics can reduce noise at high resolutions. The technique has been applied to both OCT and scanning laser ophthalmoscopy. Both Drexler and Werner have applied this technique to ultra-high resolution OCT with impressive results, especially in the imaging of the photoreceptor outer segments.^{21,32,36-38} Adaptive optics OCT has also shown impressively that it is possible to perform functional assays, so-called "optophysiology," which relies on measuring the backscatter produced by a light flash on the retina.³⁹ The potential of this technology for use in glaucoma and retinal ganglion cell assessment is under development.

Indeed, the major drawback in all OCT methodologies appears to be that the low contrast of the retinal ganglion cells and retinal nerve fiber layer makes them inherently difficult to image compared to the photoreceptors. In the future, software developments aimed specifically at assessing retinal nerve fiber layer or retinal ganglion cell quantification may allow OCT to assist in providing new clinical end points in clinical glaucoma research, but significantly more validation will need to be done first. There is a clear need for more sophisticated analysis. To this end, Fujimoto has urged that an open-access database be

made available containing OCT images acquired from anonymous patients with glaucoma and to which interested experts could assess their own analytical methods, rather like the NIH sponsored Biological Biochemical Image Database.⁴⁰ However, this method cannot be used to visualize individual retinal ganglion cells, and it is recognized that further development is needed to establish the capability of this method in glaucoma.

Spectral Domain Phase Microscopy

Spectral domain phase microscopy (SDPM), an OCT-based imaging technique, is an extension of Fourier domain OCT with spectral interferometry with a common-path geometry to depth-resolve nanometer-scale axial displacement and motion in living cells with millisecond temporal resolution.^{41,42} Simplistically, it allows the monitoring of the phase of the fringes in coherence length. Quantitative phase microscopy allows depth measurement depending on where the phase is measured from. For example, Izatt has demonstrated that the thickness of a coverslip can be measured to a resolution of less than one atom using SDPM.⁴³ High-speed cross-sectional SDPM is much faster, with 40 images acquired per second. It has been used to show spontaneously beating cardiac cells and migrating pseudopodia of moving cells in culture.

Izatt's research group has shown intricate details of retinal images using SDPM in vivo.^{44,45} Due to the rapid acquisition of images, motion artifact is eliminated, so photoreceptors, separate retinal pigment epithelium, Bruch's membrane, outer limiting membrane, and choriocapillaris may all be studied in great depth. For example, changes associated with photo-bleaching may be detected as a change in the index of refraction of photoreceptor cone outer segments.

SDPM has also been used for monitoring neuronal activation, with its ability to look at small displacements.⁴⁶ Of great potential in glaucoma is its ability to assess the swelling of individual axons accompanying action potential propagation at levels of 20-nm displacement. As with ultra-high resolution OCT and Fourier domain OCT, problems currently associated with this technology lie in dealing with the large data sets produced and the need for disease-orientated automated analysis.

High-Resolution Functional Imaging of the Retina

This modified fundus camera uses a stroboscopic flash to capture small videos comprising 8 frames of retinal images 20 to 500 msec apart. Videoanalysis of changes over time enables assessment of blood flow and functional signals.⁴⁷ Visualization of movement of single blood cells allows creation of an enhanced retinal vascular map and measurement of blood flow. Grinvald has suggested that there is a 40% reduction in blood velocity in patients with glaucoma compared to control subjects (A. Grinvald, PhD, personal communication, September 2007).

Another application of this technique is its use as a functional assay. By projecting an illuminated stimulus on to the retina, a “darkening” signal is observed with infrared light in areas of retinal stimulation and a “bright” signal in areas of activation.

A recent finding has been a “nerve fiber” signal arising from the activated area radiating to the optic disc. This follows the pattern of the arcuate fibers in the papillomacular bundle. On functional activation with the illuminated stimulus, it appears that there is a “darkening” around the optic disc in patients with glaucoma. The significance of this has not yet been elucidated, but it may offer another potential imaging parameter in glaucoma.

Magnetic Resonance Imaging

Functional magnetic resonance imaging (MRI) and enhanced MRI are commonly used in the assessment of central nervous system disease. Research⁴⁸⁻⁵⁰ has shown that retinal functional MRI could show functional responses in a cat. This technique has several applications relevant to glaucoma.⁵¹ Duncan et al.⁵² have shown it possible to assess neuronal activity associated with glaucoma using functional MRI. In patients with glaucoma who have definite visual field defects (using standard white-on-white perimetry and short wavelength automated perimetry), scotoma mapping with a checkerboard stimulus correlated positively ($r = 0.91$, $P = .01$) with functional MRI arterial spin labeling and pulse sequence diagrams. They have also correlated vision loss in glaucoma with alterations in cerebral blood flow in the V1 area of the visual cortex.

Manganese-Enhanced MRI

In animal models, manganese injections reveal retinal cellular metabolic activity through the ability of manganese to serve as a calcium ion surrogate.⁵³ Berkowitz et al.⁵⁴ and Luan et al.⁵⁵ have measured intraretinal thickness and ion channel permeation in vivo and, in different glaucoma models, demonstrated it possible to measure changes in anterior chamber angle, ciliary body area, optic nerve head width, and eye circumference.

Using manganese-enhanced MRI, Ellis-Behnke et al.⁵⁶ and Guo et al.⁵⁷ have successfully assessed the progression of functional regeneration of the visual system in vivo in a hamster optic tract regeneration model using a self-assembling peptide nanofiber scaffold to facilitate reconstruction of the tissue substrate that supports regeneration across the tissue disruption. Longitudinal follow-up of the model with sequential manganese-enhanced MRI preoperatively and immediately postoperatively and 2 and 4 weeks postoperatively revealed visibly discernible regenerating axons in the superior colliculus of treated animals after 2 weeks. It is suggested that manganese-enhanced MRI may have the potential to measure the impact of regenerative treatments in optic nerve diseases such as glaucoma prior to any signs of functional return of behavior.

MRI Imaging of Cell Death and Cell Tracking

One of the better methods of detecting apoptosis in vivo uses the highly specific binding of synaptotagmin I to phosphatidylserine. Superparamagnetic iron oxide labeling of the C2 domain of synaptotagmin I has been previously used with MRI.⁵⁸ Neves et al.⁵⁹ and Dunning et al.⁶⁰ have used the C2A domain conjugated to nanoparticles to demonstrate tumor response to treatment. A recent advance has been the use of gadolinium-tagged GST-C2, although the group is engineering derivatives of the agent to further increase specificity and sensitivity. Single cell visualization is not yet possible with this technique.^{58,61-63}

However, a secondary area of research has been the use of MRI to track individual cells labeled with different nanoparticles in vivo. This has included tracking stem cells following their implantation into the brain and spinal cord.

Array Tomography

Array tomography uses modern optical fluorescence and electron microscopy to perform 3D reconstruction of immunofluorescence-stained serial ultrathin (50 to 200 nm) sections.⁶⁴ Tissue sections are cut after embedding in a hydrophilic acrylic resin. Continuous ribbons of hundreds of serial sections on a glass slide form a two-dimensional array, which is then stained using labeled antibodies and/or other reagents. Fluorescence microscopy of stained sections is then performed, followed by alignment and collation of individual two-dimensional section images into volumetric image stacks. The method is even more exciting because large numbers of immunostains through repeated cycles of staining, imaging, and stripping individual array slides may be performed without loss of antigenicity.

This method has been used for high-throughput single-synapse analysis of mouse cerebral cortex, using synaptic molecular markers such as synapsin-I, GABA, Vglut1, Vglut2, Vglut3, and PSD-95. Smith et al. have computed large differences in synaptic density varying from 0.7 synapses per cubic millimeter in the somatosensory mouse cortex to 0.4 synapses per cubic millimeter in layer VI and 1 synapse per cubic millimeter in layer I.⁶⁵ Although this method is histological, it offers great potential in elucidating retinal neuronal anatomy in glaucoma.

Detection of Apoptosing Retinal Cells

Detection of apoptosing retinal cells (DARC) uses fluorescently labeled annexin V and fluorescent ophthalmoscopy^{6,7} to enable direct visualization of retinal ganglion cell death. Using glaucoma-related experimental models, it is a useful, noninvasive real-time imaging technique to visualize single retinal ganglion cells undergoing apoptosis in vivo.^{7,66} Apoptosis is an early cellular process in glaucoma, preceding the exponential rate of retinal ganglion cell loss responsible for irreversible blindness. DARC enables the longitudinal study of glaucomatous cellular disease firsthand, which has not previously been possible.⁶ It promises to be an invaluable tool for this condition, not only in the diagnosis and screening of patients, but also in the tracking of disease and the rapid and objective assessment of potential therapies.

DARC has already been used to assess neuroprotective strategies in experimental models.⁵ It is hoped that in addition to helping diagnose early disease, assess progression, and monitor response to treatment in patients with glaucoma, it may also serve as a new method of assessing central nervous system degeneration. In short, DARC may provide the biomarker that we have been searching for and undoubtedly need in glaucoma.

Three-Dimensional Histomorphometry of the Optic Nerve Head

Optic nerve head cupping in glaucoma has been assumed to be a manifestation of prelaminar neural tissue thinning combined with thinning and deformation of the lamina cribrosa within

the neural canal.⁶⁷ Burgoyne used 3D histomorphometric reconstruction of perfusion fixed optic nerve head tissues to show that an early sign of glaucoma is in fact thickening, not thinning, of the prelaminar neural tissues and lamina.¹¹ This could potentially be a new target for clinical 3D OCT optic nerve head imaging in patients at risk for the onset or progression of glaucomatous damage, as early data by Werner et al. suggest.^{68,69}

Multiphoton Microscopy of the Optic Nerve Head

The development of femtosecond lasers and their ability to interact with different tissues to induce two-photon fluorescence signals, with second harmonic generated signals and laser-induced optical breakdown, has been increasingly recognized as a powerful tool in ophthalmology. Jester has shown their use in determining corneal stromal lamellar organization, optic nerve head structure, and ocular biomechanical properties.⁷⁰

Second harmonic generated signals imaging microscopy provides a new approach to 3D evaluation of the collagen structure of the eye, and holds promise in determining biomechanical strength and rigidity of the cornea, retina, and optic nerve head. Recent studies have demonstrated that second harmonic generated signals from collagen allow for direct optical imaging of the lamina cribrosa.⁷¹ This structure is believed to be important because retinal ganglion cell axonal transport blockade in glaucoma (according to the mechanical theory) is believed to occur at the level of the lamina cribrosa.^{72,73} It appears that the collagen signal at the optic nerve head is reduced in glaucoma. In addition, there is increasing evidence that laminar pore size is altered in glaucoma, with peripheral and midperipheral pores being more susceptible to collapse under elevated intraocular pressure.

Two-Photon Microscopy

Using the principles of two-photon imaging, with second harmonic generated signals, and two-photon excited fluorescence imaging explained above, a two-photon ophthalmoscope based on the architecture of a conventional fast scanning laser ophthalmoscope has been developed.⁷⁴ Two-photon excited fluorescence potentially offers multiple advantages compared to single-photon fluorescence microscopy, including intrinsic 3D resolution, larger sensing depth, and reduced photodamage.

Several studies have confirmed that endogenous fluorophores in the human retina alter with age and disease. Two-photon excited fluorescence provides an opportunity to see this more clearly, with evidence that single lipofuscin granules accumulating on the retinal pigment epithelial cells and the retinal nerve fiber layer can be visualized in postmortem human donor eyes.⁷⁴⁻⁷⁶

Conclusion

These are exciting times in ophthalmic imaging, with great prospects of new clinical end points being defined. By providing objective, quantifiable, and relevant measurements, it is hoped that we will soon have a reliable, imaging-based parameter that can be used for screening, assessment of treatment, and clinical guidance in glaucoma. We are on the brink

of developing a new generation of tools that will provide us with noninvasive methods of determining neurodegenerative processes in glaucoma.

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