In Vivo Imaging of Microscopic Structures in the Rat Retina

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PURPOSE. The ability to resolve single retinal cells in rodents in vivo has applications in rodent models of the visual system and retinal disease. The authors have characterized the performance of a fluorescence adaptive optics scanning laser oph-thalmoscope (fAOSLO) that provides cellular and subcellular imaging of rat retina in vivo.

METHODS. Enhanced green fluorescent protein (eGFP) was expressed in retinal ganglion cells of normal Sprague-Dawley rats via intravitreal injections of adeno-associated viral vectors. Simultaneous reflectance and fluorescence retinal images were acquired using the fAOSLO. fAOSLO resolution was characterized by comparing in vivo images with subsequent imaging of retinal sections from the same eyes using confocal microscopy.

RESULTS. Retinal capillaries and eGFP-labeled ganglion cell bodies, dendrites, and axons were clearly resolved in vivo with adaptive optics. Adaptive optics correction reduced the total root mean square wavefront error, on average, from 0.30 μ m to 0.05 μ m (measured at 904 nm, 1.7-mm pupil). The full width at half maximum (FWHM) of the average in vivo line-spread function (LSF) was approximately 1.84 μ m, approximately 82% greater than the FWHM of the diffraction-limited LSF.

Conclusions. With perfect aberration compensation, the in vivo resolution in the rat eye could be approximately $2\times$ greater than that in the human eye because of its large numerical aperture (~0.43). Although the fAOSLO corrects a substantial fraction of the rat eye's aberrations, direct measurements of retinal image quality reveal some blur beyond that expected from diffraction. Nonetheless, subcellular features can be resolved, offering promise for using adaptive optics to investigate the rodent eye in vivo with high resolution. (*Invest Ophthalmol Vis Sci.* 2009;50:5872-5879) DOI:10.1167/iovs.09-3675

N aturally occurring, transgenic, and knockout rodent models are instrumental in the study of retinal disease mechanisms and in the development of treatments for human retinal

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dystrophies. To date, the majority of studies using rodent disease models rely on retinal histopathology to follow disease progression and the effect of candidate therapies. Histopathology yields high-resolution images and morphometric estimates of surviving retinal cells¹; however, the major drawback of this approach is that it does not allow longitudinal studies in the same animals.

In vivo imaging of the rodent retina offers the possibility of visualizing disease processes and progression in individual animals and of reducing the effects of animal-to-animal variation, background lighting, and genetic background. For example, it has been shown that in various neurodegenerative diseases, substantial modifications in the morphology of axons and dendrites can take place well before cell death.²⁻⁸ These observations highlight the need to develop high-resolution in vivo imaging techniques capable of resolving subcellular structures (such as individual axons and dendrites) in rodent eyes.

The resolution of in vivo imaging is limited by the optical quality of the rodent eye. Compared with the human eye, rodent eyes have smaller axial lengths, higher optical powers, larger average refractive errors, and larger numerical apertures (NA; Table 1^{9-20}). Rat eyes typically have a large hyperopic refractive error. Retinoscopy measurements have shown refractive errors in the range of +5 to +15 D in albino rats, with a strong dependence on the strain (Irving EL, et al. *IOVS* 2005;46:ARVO E-Abstract 4334). A dilated rodent eye also has a larger numerical aperture than a dilated human eye. Thus, in theory, one could resolve smaller retinal features with a perfect correction of the eye's aberrations in a dilated rodent eye than in a dilated human eye.

Many studies have used single-photon fluorescence microscopy, fundus photography, two-photon microscopy, confocal microscopy, or scanning laser ophthalmoscopy (SLO) to image the living rodent retina, allowing the visualization of structures such as blood vessels, capillaries, nerve fiber bundles, photoreceptors, retinal ganglion cells (RGCs), retinal pigment epi-thelial (RPE) cells, and microglial cells.²¹⁻³⁹ Fluorescently labeled RGCs have been imaged in the rodent retina in vivo over a wide field,^{21-28,36,38,39} with some studies showing the appar-ent loss of ganglion cells in diseased retina.^{23,24,26,27,38,39} One report recently used a confocal laser scanning microscope to image ganglion cells and processes in vivo in transgenic mice that expressed yellow fluorescent protein (YFP) in a small subset of RGCs.³³ Resolution in all these studies could be improved by correcting the eye's aberrations with adaptive optics so that many fine features that could previously be resolved only in excised retina could now be imaged in vivo. Adaptive optics ophthalmoscopes have enabled near diffraction-limited imaging of cellular structures (such as individual photoreceptors, ganglion cells, and RPE cells) in living human and nonhuman primates, 40-44 and the resolution of subcellular features (such as ganglion cell axons and dendrites) in living nonhuman primates.^{42,43} Recently, Biss et al.³¹ fluorescently imaged mouse capillaries and microglia cells using an adaptive optics biomicroscope, showing that some of the benefits of adaptive optics found in primates can be realized in rodent eves.

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TABLE	1.	Ocular	Parameters	for	Human,	Rat,	and	Mouse	Eyes
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	Average Axial Length (mm)	Total Power (D)	Average Refractive Error (D)	Numerical Aperture (NA)*	Theoretical Maximum Lateral Resolution (µm)†	
Human Rat Mouse	$\begin{array}{c} \sim 23.5\text{-}24^{9,10} \\ \sim 6.1^{16} \\ \sim 3.3^{17} \end{array}$	$\sim 60 \\ \sim 300^{16} \\ \sim 560^{18}$	$\sim 0 \text{ to } +1^{11-15}$ +5 to +15‡ +7 to +15 ^{17,19,20} §	$\sim 0.20 \\ \sim 0.43 \\ \sim 0.49$	1.68 0.78 0.68	

* Calculated for dilated human, rat, and mouse pupil sizes of 6.8 mm, 3 mm, and 2 mm, respectively.

† Theoretical maximum lateral resolution calculated for 550-nm light.

‡ Irving EL, et al. IOVS 2005;45:ARVO E-Abstract 4334.

§ Beuerman RW, et al. IOVS 2003;44:ARVO E-Abstract 4338.

We describe here a fluorescence adaptive optics scanning laser ophthalmoscope (fAOSLO) for the rat eye. We show that cellular and subcellular features in the rat retina, such as fine capillaries and individual fluorescently labeled ganglion cell dendrites and axons, can be imaged. The in vivo resolution of our instrument was subsequently determined via confocal images of flatmounts of the enucleated retinas.

METHODS

Normal Sprague-Dawley rats from 2 to 21 months of age were used in this study. All animals were handled according to the ARVO Statement for the Use of Animals in Ophthalmic and Vision Research and the guidelines of the University Committee on Animal Resources at the University of Rochester.

eGFP Labeling of RGCs

Intravitreal injections (3-5 μ L [0.5-5 \times 10¹³ vg/mL]) of adeno-associated serotype 2 (AAV2) viral vectors containing a human synapsin promoter were used to express enhanced green fluorescent protein (eGFP) in ganglion cells of normal adult Sprague-Dawley rats. Vectors were produced and purified as described by Zolotukhin et al.45 Rats were anesthetized using a ketamine (~65 mg/kg) and xylazine (~8 mg/kg) cocktail, and intravitreal injections were made through the sclera using a Hamilton syringe fitted with a 1-cm, 32-gauge needle (described in detail by Greenberg et al.⁴⁶). Retinal ganglion cells displayed visible in vivo eGFP fluorescence approximately 4 weeks after injection that persisted for the life of the animal. Fluorescence fundus photographs were acquired with a modified fundus camera47 or a wide-field digital imaging camera (RetCam; Clarity Medical Systems, Inc., Pleasanton, CA) to image ganglion cell eGFP labeling before high-resolution fAOSLO imaging. We estimate that more than 90% of the retinal ganglion cells were transduced by the AAV2 viral vectors because the entire retina was contacted by the AAV2 vectors after an intravitreal injection.

Preparation for In Vivo AOSLO Imaging

Rats were anesthetized with isoflurane gas (2%-3%) and placed on a heating pad before in vivo imaging. Pupils were dilated with one drop of 2.5% phenylephrine (Neo-Synephrine; Bayer, Pittsburgh, PA) and one drop of 0.5% tropicamide. A 0 to +20 D rigid contact lens (Unicon Corporation, Osaka, Japan) was placed on the eye to maintain corneal hydration and to reduce refractive error during in vivo fAOSLO imaging. Slit lamp imaging and retinoscopy were used to determine the base curve and refractive power of the contact lens required for each eye.

Rats were stabilized for imaging on a custom stereotaxic apparatus with ear bars. A three-axis translation stage was used to align the rat's pupil with the exit pupil of the fAOSLO. A goniometer and a rotation mount (with their centers of rotation centered in space on the eye's pupil) were used to rotate the angle of the entrance beam to image different retinal locations. Large-scale features (such as blood vessels) observed in the previously acquired fluorescent fundus photographs (see Fig. 3) were used as landmarks to navigate to desired retinal locations and to track the same location over multiple imaging sessions.

Rat Confocal fAOSLO

High-resolution retinal images were obtained using a modified fAOSLO initially developed for imaging human and nonhuman primates.⁴² The scanning instrument (Fig. 1) incorporated an adaptive optics system consisting of a Shack-Hartman wavefront sensor and a 4μ m stroke MEMS deformable mirror (Boston Micromachines Corporation, Boston, MA). A removable fold mirror was inserted into the system and used to divert the imaging beam to a separate rodent arm either with a pair of spherical mirrors (M11, FM) that demagnified the pupil size to 3.5 mm or to an achromatic triplet lens (not shown) that demagnified the pupil sizes for the 3.5- and 2.1-mm pupil sizes were 5.5° and 9°, respectively.

A 794-nm super luminescent diode (SLD; Superlum, Cork, Ireland) was used for reflectance imaging, and the 488-nm line from an argon/krypton laser (Melles Griot, Carlsbad, CA) was used for fluorescence excitation. Wavefront sensing of the rat eye's aberrations was performed using a 904-nm laser diode (Thorlabs Inc., Newton, NJ). The maximum permissible exposure (MPE) for using these three sources simultaneously in the rat eye was calculated assuming that the rat retina had the same sensitivity as the human retina. These calculations also included a scaling factor that incorporated the differences in the spot size and retinal illuminance at the rat retina because of its higher numerical aperture (when compared to the human eye). Less than 80 μ W, 350 μ W, and 20 μ W were used for the 904-nm laser diode, 794-nm SLD, and 488-nm laser, respectively. In aggregate, these combined powers were less than the MPE for a 60-minute exposure time and 3° field size according to the ANSI guide for the safe use of lasers,48 and the "translation" of the ANSI standard for typical devices by Delori et al.49

For in vivo imaging sessions, wavefront sensing and adaptive optics correction were performed over a 1.7- to 3.5-mm pupil size (depending on the magnification) at a rate of 15 Hz. Residual spherocylindrical errors not corrected by the contact lens were corrected by placing trial lenses in front of the rat eye or in a conjugate system pupil plane (TL). To image different retinal layers, the imaging beam was defocused from the wavefront sensing plane using spherical trial lenses or by changing the focus of the deformable mirror. This defocus also incorporated the chromatic aberration between the imaging and wavefront sensing wavelengths.

Image Acquisition and Analyses

Single in vivo fluorescence frames typically had very low signal-to-noise ratios. Therefore, several fluorescence frames were summed to obtain an image with a high signal-to-noise ratio. To account for eye movements between different frames, images were registered using the shifts calculated from simultaneously acquired reflectance images.⁴² A cold mirror (CM2) was used to separate the visible and infrared light for simultaneous fluorescence and reflectance imaging. An avalanche photodiode (Perkin Elmer, Vaudreuil, QC, Canada) was used for reflectance detection at 794 nm, and a photomultiplier tube (H7422-40;



the rat eye, based on the system previously described by Gray et al.42 L. lenses; M, spherical mirrors; FM, fold mirrors; RFM, removable fold mirror; CM, cold mirrors; FC, fiber collimators: WFS, wavefront sensor: DBS, dichroic beam splitters; CP, confocal pinholes; SLD, fiber coupled 794-nm super luminescent diode; LDB, laser diode beacon; BS 1, 90/10 beam splitter; DM, MEMS deformable mirror; TL, trial lens plane; PBS, pellicle beam splitter; VS, vertical scanner (27 Hz); HS, horizontal scanner (15.1 kHz): F, bandpass fluorescence filter: PMT, photomultiplier tube; APD, avalanche photodiode; LEI Box, scanner control electronics. Adapted with permission from Gray DC, Merigan W, Wolfing JI, et al. In vivo fluorescence imaging of primate retinal ganglion cells and retinal pigment epithelial cells. Opt Express. 2006;14: 7144-7158. © 2006 Optical Society of America.

Hamamatsu, Shizuoka-ken, Japan) was used for fluorescence detection of eGFP. Removable confocal pinholes were placed at retinal conjugate planes in front of both detectors. Typical pinhole sizes ranged from 4.1 to 6.2 Airy disc diameters for reflectance imaging and 2.3 to 7.0 Airy disc diameters for fluorescence imaging. Images were acquired at a rate of 27 Hz.

As described previously, the maximum achievable imaging field size was 9°. Although the resolution of this image was reduced, likely because it exceeded the isoplanatic patch size in the rat eye, the larger fields of view were helpful for generating quick montages to identify landmarks or to image larger features. Larger montages of nearby retinal locations were created by registering multiple images together using a custom-written algorithm or by manually registering images using graphics editing software (Adobe Photoshop; Adobe Systems Inc., San Jose, CA). To more easily identify capillaries within the reflectance images, we computed the standard deviation of each pixel in the stabilized, registered images (Burns SA, et al. IOVS 2008;49:ARVO E-Abstract 4512) using ImageJ software (developed by Wayne Rasband, National Institutes of Health, Bethesda, MD; available at http://rsb.info.nih.gov/ij/index.html). Changes in reflectance from blood flow through the capillaries will result in greater variability (or a higher standard deviation) in the reflectance from the capillaries compared with the more static surrounding tissue.

To estimate the physical size of structures in the in vivo retinal images using its angular dimensions, we assumed that our rat eyes had the same focal length and vitreous refractive index as Hughes' rat schematic eye.16 The retinal spatial-to-angular conversion was calculated to be 58.1 μ m/deg. fAOSLO images in this study were collected over a 3° or 4.7° field of view, corresponding to a physical width of 174.3 µm or 273.1 µm, respectively, on the retina.

Characterization of the In Vivo **Transverse Resolution**

Rats were euthanatized after multiple in vivo imaging sessions. Retinas were removed and placed as wholemounts on slides with coverslips and covered in mounting medium (Vectashield; Vector Laboratories, Burlingame, CA). Wholemount images were acquired using a confocal microscope (Zeiss LSM510; Carl Zeiss, Inc., Oberkochen, Germany). Through-focus stacks were taken at the same locations as the in vivo images using a $10 \times (0.30 \text{ NA})$ and $40 \times (1.2 \text{ NA})$ microscope objective, and maximum intensity projection images were generated for each stack. The pinhole size in the confocal microscope was adjusted to match the in vivo imaging parameters. Ex vivo images acquired with the 1.2 NA objective were taken to represent the true size of the object.

The in vivo transverse resolution of the fAOSLO was determined by calculating the in vivo line spread function (LSF). First, average crosssectional profiles were measured across the same individual dendrites in the in vivo and ex vivo images. Both in vivo and ex vivo profiles were normalized with regard to the background level. The in vivo LSF was calculated by deconvolving the ex vivo dendrite profile from the corresponding in vivo dendrite profile. The in vivo resolution of the fAOSLO was defined as the full width at half maximum (FWHM) of the in vivo LSF.

RESULTS

In Vivo Adaptive Optics Imaging of Capillaries in Reflectance

Adaptive optics correction typically reduced the higher order root mean square (RMS) wavefront error (as measured by the Shack-Hartman wavefront sensor for the 904-nm wavefront FIGURE 2. In vivo reflectance images of the rat retina taken (a) before and (b) after adaptive optics correction. Both images are the registered sum of 200 frames. (c) Standard deviation image calculated from the registered video acquired after adaptive optics correction. Confocal pinhole diameter was 6.2 Airy disks. Scale bar, 20 µm.



sensing beacon) from ~0.5 μ m to ~0.1 μ m for a 3-mm pupil and from ~0.3 μ m to ~0.05 μ m for a 1.7- to 1.9-mm pupil in our system. (For comparison, the Maréchal criterion of $\lambda/14$ at the wavefront sensing wavelength is ~0.06 μ m.) In vivo images of retinal capillaries (Fig. 2) were acquired in reflectance after adaptive optics correction over a 1.85-mm pupil. All reflectance images correspond to the registered sum of 200 individual frames. Retinal features were difficult to discriminate before adaptive optics correction. After correcting for the rat eye's aberrations, capillaries were seen faintly in reflectance images.

The areas of blood flow can be visualized more clearly by computing the standard deviation of each pixel throughout the series of registered frames. Figure 2c shows the standard deviation image calculated from the registered sequence that produced the image in Figure 2b. Areas with high variability in their reflectance appear as bright regions in the image, whereas areas with relatively constant reflectance appear dark. The capillary network clearly emerged using this technique because of changes in capillary reflectance over time attributed to the movement of cells inside the capillary walls.

In Vivo fAOSLO Imaging of Fluorescently Labeled Ganglion Cells, Axons, and Dendrites

Before high-resolution fluorescence imaging with adaptive optics, wide-field fluorescence fundus photographs were acquired (see Methods) to verify eGFP expression had begun (Fig. 3). The ganglion cell layer was then imaged with the



FIGURE 3. In vivo fluorescence fundus photographs taken in the rat eye to verify eGFP expression before fAOSLO imaging. The rectangular image overlaid on the photograph is a montage of low-resolution images acquired with the fAOSLO. The fAOSLO montage image was composed of five low-resolution images collected over a 9° field-of-view; each image was a registered sum of 1000 frames. Both the fundus photographs and low-resolution fAOSLO montages served as maps to guide imaging over small fields with the high-resolution fAOSLO. A magnified, high-resolution image of the region denoted by the *white square* is illustrated in Figure 5.

fAOSLO (Fig. 4). Large ganglion cell bodies could be observed in vivo without aberration correction (Fig. 4a), consistent with earlier studies.²¹⁻²⁸ Adaptive optics correction of the rat's aberrations enabled us to readily distinguish individual RGC bodies that were difficult to differentiate before adaptive optics correction (Figs. 4a, b, arrows). In addition, adaptive optics allowed the clear visualization of RGC axons and dendrites (Fig. 4b). Using retinal landmarks, we were able to return to an identified retinal location in the same rodent, permitting the longitudinal documentation of individual RGCs. Figure 4c shows a retinal image acquired at precisely the same retinal location in an individual rat that was imaged again 3 months later. The confocal pinhole size used for obtaining this image (2.3 Airy disc sizes) was one-third the size of that used for obtaining the image in Figure 4b; thus, fewer photons were collected for the image in Figure 4c. Although defocus might not have been optimized in this image, the same cell bodies and processes were resolved.

Direct Comparison of In Vivo Images to Ex Vivo Images

We compared the structure of individual RGCs observed in fAOSLO images obtained in vivo with the RGC structure seen in histologic preparations of enucleated retina from the same eyes with confocal microscopy. Figure 5 directly compares in vivo and ex vivo images of an RGC with a large diameter cell body. This is likely a giant cell (or an RGA1 cell according to Huxlin and Goodchild's classification).⁵⁰ Figure 5a shows the area inside of the white square overlaid on the low-resolution fAOSLO montage from Figure 3. The in vivo image is a maximum intensity projection image generated from three separate in vivo images taken at different depths (focus step is 1.1 D or 16.3 μ m). Ex vivo images are maximum intensity projection images generated from a through-focus stack of 63 images in the ganglion cell layer. This comparison exaggerates the difference between the in vivo and ex vivo images due to both the finer focus step and larger focus range in the ex vivo image stack. However, the in vivo 0.25 NA image recovers almost all the information in the ex vivo 0.3 NA image.

Characterization of In Vivo Transverse Resolution

In vivo imaging resolution was characterized by comparing the in vivo images with corresponding ex vivo histologic images. The in vivo LSF was computed over the four locations indicated by the four gray rectangles in Figures 5a and 5c. Figure 6a shows the average profiles measured across the same dendrite imaged in vivo (open circles with dashed line) and ex vivo using a 1.2-NA microscope objective (filled circles with solid line) from the rightmost gray rectangle in Figures 5a and 5c. The normalized in vivo LSF (Fig. 6b) was calculated by deconvolving the ex vivo dendrite profile from the corresponding in vivo dendrite profile. This calculation assumes that the LSF of the 1.2-NA microscope objective is small and that the ex vivo



FIGURE 4. In vivo fluorescence images (excitation at 488 nm, peak emission at 507 nm) of rat retinal ganglion cells, dendrites, and axons labeled with eGFP. (a) Image taken before adaptive optics correction with a best correction of defocus and astigmatism. Some (but not all) cell bodies can be resolved, whereas subcellular features are blurred. (b) Image with adaptive optics correction. All cell bodies can be differentiated, and subcellular processes are evident. (c) In vivo image of the same retinal location shown in (b) taken 3 months later. The same cellular and subcellular features can be resolved. Adaptive optics correction was performed over a 1.7-mm pupil. All images are the registered sum of 1000 frames. All images were contrast enhanced identically for display purposes. Scale bar, 20 μ m.

image reveals the true dimensions of the dendrite. (This assumption is reasonable because the diffraction-limited LSF of the microscope is approximately 0.21 μ m, which is five times smaller than the LSF estimated for diffraction-limited in vivo imaging. Simulations show that when imaged by a 1.2-NA objective, dendrites with FWHMs from 1.3 to 1.9 μ m broaden by <5%.) For this location, the FWHM of the in vivo LSF was 1.77 μ m. The average FWHM of the in vivo LSFs calculated at all four locations was 1.84 ± 0.30 μ m (average ± SD), approximately 82% larger than the FWHM of the diffraction-limited LSF (1.01 μ m).

DISCUSSION

Challenges of In Vivo Imaging of the Rodent Eye Using Adaptive Optics

It is challenging to achieve diffraction-limited imaging for the rat eye because of its small size, high optical power, and large refractive error. Given the high optical power of the rodent eve, a large defocus value is needed for sectioning the retina. The total optical power for the human eye is ~ 60 D and for the rat eye it is \sim 300 D, yet the rodent retina is comparable in average thickness to that of human (typically 170 μ m vs. 250 μ m, respectively).^{16,51} Whereas only ~0.66 diopter is required to shift the focal plane in the human from the anterior to the posterior retina, an ~ 11.5 diopter change in optical power is required in the rat. Considering the different pupil sizes inherent in different species, the amount of Zernike defocus (in micrometers) needed to section the whole retina is less than 1 μm for the human eye and 5 μm for the rat eye. The large refractive error of the rodent eye poses another challenge for in vivo cellular imaging. We used trial lenses and contact lenses to compensate for spherocylindrical errors, but using a larger stroke deformable mirror for refractive correction would provide a faster solution. Another issue to be considered when imaging with multiple wavelengths is the increased amount of



FIGURE 5. Direct comparison of in vivo and ex vivo rat retinal images. (a) In vivo image in a rat retina taken with adaptive optics correction over a 0.25 NA. (b) Ex vivo histologic image acquired using a confocal microscope with a 0.3 NA objective. (c) Ex vivo histologic image acquired using a confocal microscope with a 1.2 NA objective in which details of the cell soma, dendrite, and axon structures can be seen. The in vivo image is a maximum intensity projection image generated from three separate in vivo images taken at different depths, each of which is a registered sum of 1000 frames. Both ex vivo images are maximum intensity projection images generated from a focus stack of 63 images of the ganglion cell layer. (a, c, *gray rectangles*) Locations in which we characterized the in vivo resolution. All images have been rescaled and contrast enhanced for display purposes. Scale bar, 20 μ m.

In vivo vs. ex vivo average dendrite profile

Expected vs. calculated in vivo LSF



FIGURE 6. Characterization of in vivo transverse resolution. (a) Average normalized cross-sections of a dendrite imaged in vivo with a 0.25 NA objective (*open circles, dashed line*) and ex vivo with a 1.2 NA objective (*filled circles, solid line*). (b) Calculated in vivo LSF compared with expected theoretical LSF for a 0.25 NA objective. *Solid line*: calculated in vivo LSF for a 0.25 NA objective with a FWHM of 1.77 μ m. For comparison, the expected theoretical LSF for a 0.25 NA eye is plotted (*dashed line*) with a FWHM of 1.01 μ m.

chromatic aberration in the rat eye compared with that of the human eye; the chromatic aberration between 475 nm and 650 nm in the rat eye is reported to be 5.8 D.⁵² Last, but not least, wavefront sensing using reflective infrared light (which returns primarily from the photoreceptors) can cause additional uncertainties for effective adaptive optics correction when imaging retinal layers away from the photoreceptor layer. For example, in the rat, the ~11.5 D separation between the ganglion cell and photoreceptor layers could require modifications of other aberrations, such as spherical aberration, for best correction. Measurements of any variation in the wave aberration with different focal planes throughout the retina have yet to be made.

Careful animal handling is also important to achieve successful images. For fAOSLO imaging of the rat retina, we have found that isoflurane provides a better, more stable plane of anesthesia than ketamine and xylazine. The morbidity rates after anesthesia are lower, fewer eye movements are observed in the high-magnification images, and the duration of the anesthesia can be longer (more than 3 hours vs. 30 minutes) when using isoflurane. A heating pad helps to keep the animal warm and prevent transient, temperature-related cataract formation for imaging sessions longer than 1 hour. Using contact lenses to keep the eye hydrated eliminates the need to manually blink the eye or constantly hydrate the eye with drops and avoids distortions in the wavefront caused by degradations in the tear film or nonuniformities in the distribution of the hydrating drops. A contact lens also helps to reduce the lower order aberrations of the eye. We found that the spherical refractive power and corneal curvature of our Sprague-Dawley rat eyes changed dramatically with age. Their spherical refractive power decreased from 10 to 20 D to approximately 5 D as they matured. Therefore, it is important to carefully select and match the base curve, diameter, and power of the contact lens to the eye being imaged. Poor-fitting contact lenses may degrade the spots in the wavefront sensor images, resulting in poor adaptive optics corrections and image quality.

In Vivo Imaging of Rat Capillaries in Reflectance

Several studies have imaged rodent capillaries using fluorescein angiography,^{29-31,34,35} and Paques et al.³⁰ attempted to image

mouse capillaries in reflectance with an SLO. In the present study, we imaged rat capillaries in reflectance with the fAOSLO. However, it is difficult to clearly differentiate all capillaries. The reflectance images shown in Figures 2a and 2b appear blotchy with bright and dark areas. The exact origin of this blotchy appearance is not yet well understood, but they are stable features of these retinal images and may be related to out-of-focus light from other retinal layers.

The standard deviation image processing technique highlights areas with high variability in the reflectance image, such as regions with blood flow. The combination of fAOSLO imaging and standard deviation image processing enables visualization of small capillaries in reflectance with image quality comparable to that of fluorescein angiography. This method presents a useful noninvasive alternative to performing fluorescein angiography for imaging capillaries in rodent and human eyes.

Imaging Subcellular Features of Rat Ganglion Cells In Vivo

Fluorescence imaging has been the most successful method to provide contrast for transparent retinal cells, such as ganglion cells, in living eyes. Sabel et al.²¹ and Rousseau et al.²² used a modified confocal laser scanning microscope to first visualize rat RGCs that were retrogradely labeled via injections of fluorescent latex beads into superior colliculus. Naskar et al.²³ and Thanos et al.²⁴ monitored the death of retrogradely labeled rat RGCs using conventional fluorescence microscopy. Paques et al.²⁵ visualized rat RGCs using fundus photography after retrograde transport of indocyanine green that was injected into the LGN. More recently, many in vivo studies have imaged RGCs using the SLO. Cordeiro et al.²⁶ labeled rat and primate RGCs with intravitreal injections of annexin 5 and visualized RGC apoptosis using a confocal SLO. Higashide et al.27 imaged and counted retrogradely labeled rat RGCs using an SLO. Recent reports from Leung et al.^{28,39} also used a confocal SLO to image mice RGCs that were intrinsically labeled in a strain of transgenic mice. All these studies were able to visualize ganglion cell bodies. In another recent report, Walsh and Quigley³³ were able to visualize ganglion cell bodies and their processes in a transgenic mouse that expressed YFP in a small subset of RGCs. Even though ganglion cell processes have been visualized in the mouse eye by selectively labeling a small subset of RGCs,³³ the increased resolution afforded by adaptive optics enables us to resolve ganglion cell axons and dendrites in a densely labeled retina.

The major difference between the present study and these other studies is the use of adaptive optics to increase lateral resolution and provide higher magnification images. This method will potentially enable more accurate cell counting because cells that are difficult to differentiate in dense clumps in low-resolution images can be individually distinguished using adaptive optics imaging. Adaptive optics imaging can also increase the amount of light detected through the confocal pinhole, translating into an increased axial resolution and signal-to-noise ratio (because a smaller pinhole size can be used in confocal imaging). The fAOSLO may also be used to track structures longitudinally, such as photoreceptors, capillaries, and fine features of other neurons, in the rodent retina in vivo.

Of course, many in vivo rodent retinal imaging experiments can be conducted successfully without using adaptive optics. For example, a conventional fluorescence SLO is sufficient to image larger structures, such as blood vessels,^{29,30,34} or to monitor general patterns of ganglion cell and axon bundle loss in the eye.^{26,27,39} To image ganglion cell dendrites in a sparsely labeled retina, one can use a confocal microscope.³³ To image ganglion cell dendrites, perform exact cell counts in a densely labeled retina, or resolve other subcellular features, adaptive optics and confocal imaging are very helpful. Adaptive optics provides rapid, automated correction of all the monochromatic aberrations of the eye, including both lower and higher order aberrations, that are difficult to correct using other methods.

In Vivo Transverse Resolution

To our knowledge, the present study is the first to characterize the in vivo imaging resolution in rodent eyes. Despite the fact that the residual RMS wavefront error was approximately 0.05 μ m after adaptive optics correction, the in vivo transverse LSF was found to be approximately 82% larger than expected. A number of factors may account for this difference. Our leading candidate is nonoptimized imaging defocus. As mentioned, at the location shown in Figure 5, three separate in vivo images were taken at focus steps of 16.3 μ m. In comparison, the depth of focus is calculated to be 8.0 $\mu m.$ Simulations show that a defocus value of $\pm(16.3/2)$ µm along the axial direction is consistent with the 82% error. Therefore, finer focus steps would be needed to achieve better resolution. Another possible factor is that the wavefront sensor may not be capturing all sources of image blur because wavefront sensing and imaging are performed in two different retinal layers (as previously discussed). Other possible contributing factors include accounting for eye movements within an individual frame, additional complications in wavefront sensing caused by non-common path errors, and scattering. Thus, although we have achieved an in vivo resolution in which we can resolve subcellular features such as axons and dendrites, there is promise for further resolution improvement.

In this report, the in vivo imaging resolution was characterized over a 1.7-mm pupil size (0.25 NA). We have also imaged ganglion cells and processes using a 3-mm pupil (0.43 NA) with residual RMS wavefront errors on the order of 0.1 μ m after adaptive optics correction. Although we were unable to characterize the in vivo resolution using images acquired through this 3-mm pupil size (because of insufficient labeling and contrast in these retinal images), the in vivo resolution could be higher with a 3-mm pupil (as opposed to a 1.7-mm pupil) given the larger diffraction limit. It may eventually be possible to exceed the diffraction limit imposed by the dilated pupil of the eye. Methods to achieve this improvement in retinal imaging are under development.⁵³

Future Applications

The ability to resolve subcellular features such as individual ganglion cell axons and dendrites in rodents in vivo provides us the opportunity to detect early changes in the retina on a microscopic level in disease models and to investigate fundamental questions about retinal disease pathophysiology. For example, adaptive optics imaging may help to determine whether remodeling occurs during glaucoma, whether axonal degeneration precedes dendrite degeneration, what the pattern of cell death is, and whether vascular degeneration precedes RGC death. Although we have concentrated on ganglion cell imaging in this study, this level of resolution indicates that it may be possible to use the fAOSLO to image individual Müller glial cells, RPE cells, and rod and cone photoreceptors that are fluorescently labeled in transgenic rodents^{54,55} or labeled using cell-specific adeno-associated virus (AAV) and lentiviral vectors.^{46,56–58}

The mouse is the most widely used animal model for human retinal degeneration. A similar imaging method might also be applied to image multiple cells types in normal and transgenic mice. With the current powerful molecular techniques that exist for engineering the mouse, fAOSLO imaging of mouse models has potential for investigating disease mechanisms and evaluating the efficacy of drug therapies. Finally, it may also be possible to combine fAOSLO imaging with optical methods used to image the functional activity of cells, allowing optical recordings of the activity of single retinal cells or ensembles of retinal cells in vivo.

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