Optimization of In Vivo Confocal Autofluorescence Imaging of the Ocular Fundus in Mice and Its Application to Models of Human Retinal Degeneration

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PURPOSE. To investigate the feasibility and to identify sources of experimental variability of quantitative and qualitative fundus autofluorescence (AF) assessment in mice.

METHODS. Blue (488 nm) and near-infrared (790 nm) fundus AF imaging was performed in various mouse strains and disease models (129S2, C57Bl/6, *Abca4*^{-/-}, C3H-*Pde6b*^{rd1/rd1}, *Rbo*^{-/-}, and BALB/c mice) using a commercially available scanning laser ophthalmoscope. Gray-level analysis was used to explore factors influencing fundus AF measurements.

RESULTS. A contact lens avoided cataract development and resulted in consistent fundus AF recordings. Fundus illumination and magnification were sensitive to changes of the camera position. Standardized adjustment of the recorded confocal plane and consideration of the pupil area allowed reproducible recording of fundus AF from the retinal pigment epithelium with an intersession coefficient of repeatability of $\pm 22\%$. Photopigment bleaching occurred during the first 1.5 seconds of exposure to 488 nm blue light (~ 10 mW/cm²), resulting in an increase of fundus AF. In addition, there was a slight decrease in fundus AF during prolonged blue light exposure. Fundus AF at 488 nm was low in animals with an absence of a normal visual cycle, and high in BALB/c and *Abca4*^{-/-} mice. Degenerative alterations in *Pde6b*^{rd1/rd1} and *Rbo*^{-/-} were reminiscent of findings in human retinal disease.

CONCLUSIONS. Investigation of retinal phenotypes in mice is possible in vivo using standardized fundus AF imaging. Correlation with postmortem analysis is likely to lead to further understanding of human disease phenotypes and of retinal degenerations in general. Fundus AF imaging may be useful as an outcome measure in preclinical trials, such as for monitoring effects aimed at lowering lipofuscin accumulation in the retinal pigment epithelium. (*Invest Ophthalmol Vis Sci.* 2012; 53:1066–1075) DOI:10.1167/iov.11-8767

Retinal research, including the investigation of novel therapies for retinal degenerative or neovascular diseases, relies heavily on the use of animal models. Small rodents constitute the majority of disease models that have been developed to date.¹ Therefore, assessment of the rodent retina has become increasingly important in basic and preclinical research and furthers the understanding of human retinal disease.

Histologic analysis of tissue specimens obtained after euthanasia was once the only means by which to investigate retinal structure in animal models. Recently developed retinal imaging modalities, such as confocal scanning laser ophthalmoscopy (cSLO)^{2,3} and optical coherence tomography (OCT),⁴ may now be used for high-resolution in vivo assessment of retinal structure in small animals. Repeated in vivo assessments may be performed longitudinally in the same animal, reducing variability and, potentially, the number of animals required in some experiments.

Fundus autofluorescence (AF) imaging allows noninvasive assessment of retinal disease. In humans, fundus AF findings may inform disease diagnosis, characterization, prognostication, and monitoring.⁵ Currently, a 488 nm laser light source is most commonly used for AF excitation. At this excitation wavelength, the main fluorophore in the human fundus is lipofuscin.⁶ Lipofuscin is a natural byproduct of the visual cycle and is composed of compounds that normally accumulate in the retinal pigment epithelium (RPE) with age.^{6,7} Retinal disease may be associated with abnormally increased⁸ or decreased^{9–11} 488 nm AF, specific alterations of the AF pattern,⁵ or both. Near-infrared (NIR) light using a 790 nm laser has also been used for fundus AF imaging. The AF signal at this excitation wavelength appears to originate from melanin in the RPE and choroid^{12–14}; 790 nm AF may show similar, but distinct, alterations in retinal diseases compared with 488 nm AF, possibly because of changes in the melanosome compartment.^{15–17}

A high-resolution, high-contrast image of fundus AF distribution in humans can be visualized with cSLO. The use of cSLO imaging in animal models has been described.^{2,3} Despite these pioneering advances in this field, cSLO AF imaging of the ocular fundus in mice has not been investigated systematically. Herein, we aimed to explore factors influencing fundus AF imaging in mice and to optimize the technique to be able to

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apply fundus AF imaging in a standardized, consistent, and reproducible way. cSLO imaging was subsequently used to characterize 488 nm and 790 nm fundus AF in different wild-type (WT) mice and in retinal disease models. These data provide evidence that a standardized approach to fundus AF imaging allows the recognition of disease-related changes of AF patterns (i.e., qualitative changes) and the determination of fundus AF intensity (i.e., quantitative changes) in vivo as an alternative to postmortem measurements of lipofuscin components.

METHODS

Mice

Pigmented WT 129S2/SvHsd (WT129) and C3H/HeNHsd-*Pde6b*^{rd1} (*Pde6b*^{rd1/rd1}) were purchased from Harlan Laboratories (Hillcrest, UK). WT C57Bl/6 and albino BALB/c mice were provided by the Biomedical Sciences division of the University of Oxford. Pigmented *Abca4* knockout mice (129S4/SvJae-*Abca4*^{tm1Gbt} [*Abca4*^{-/-}])¹⁸ were provided by Gabriel Travis (David Geffen School of Medicine, University of California, Los Angeles, CA). Rhodopsin knockout mice (B6.129S1-*Rho*^{tm1Pbm} [*Rho*^{-/-}])¹⁹ were a kind gift obtained under MTA from Jane Farrar (Trinity College Dublin, Ireland). Mice expressing the fluorescent reporter protein DsRed under a modified actin promoter (Tg(CAG-DsRed**MST*)1Nagy/J) were purchased from The Jackson Laboratory (Bar Harbor, ME).

Animals were kept in a 12-hour light (<100 lux)/12-hour dark cycle, with food and water available ad libitum. All procedures were performed under the approval of local and national ethical and legal authorities and in accordance with the ARVO Statement for the Care and Use of Animals in Ophthalmic and Vision Research.

Animal Preparation for Imaging Procedures

Mice were anesthetized by intraperitoneal injection of 1 mg/kg medetomidine (Dormitor 1 mg/mL; Pfizer, Sandwich, UK) and 60 mg/kg ketamine (Ketaset 100 mg/kg; Fort Dodge, Southampton, UK). Their pupils were dilated with tropicamide (Mydraticum 1%; Bausch & Lomb, Kingston-on-Thames, UK) and phenylephrine (phenylephrine hydrochloride 2.5%; Bausch & Lomb) eyedrops. A custom-made contact lens was used for all recordings (except where stated otherwise) to prevent corneal desiccation and subsequent cataract formation and to improve image quality (PMMA mouse lens, back optic zone radius of 1.7 mm, total diameter of 3.2 mm, center thickness of 0.4 mm, straight sides; Cantor and Nissel, Brackley, UK). Lubricating eyedrops (Hypromellose BPC 0.3%; Matindale Pharmaceuticals, Romford, UK) were used as viscous coupling fluid between the contact lens and the cornea. For image acquisition, the animal was placed on a platform mounted on the chin rest of the imaging device so that its eyes were positioned approximately at the level of the marking for a human patients' eye position. If prolonged imaging is required, a heat mat can be used to maintain body temperature. In selected cases, mice with dilated pupils can be assessed with the cSLO with only brief physical restraint, without general anesthesia and without a contact lens placed on the cornea. For consistent recordings across animals allowing gray-level (GL) analysis, imaging was performed under anesthesia throughout this study.

Fundus Imaging Using a Confocal Scanning Laser Ophthalmoscope

cSLO imaging was performed with a device commercially available for human fundus imaging (Spectralis HRA; Heidelberg Engineering, Heidelberg, Germany).²⁰ Fluorescence was excited using laser diodes centered at approximately 488 nm and 788 nm. Emission was recorded between 500 and 700 nm or above 805 nm, respectively. The beam powers were approximately 280 μ W and 2.6 mW, respectively.

The NIR reflectance mode (820 nm laser) was used for camera alignment to obtain an evenly illuminated fundus image and to focus on the confocal plane of interest. A confocal reference plane of high reflectivity could be consistently identified in the outer retina using the NIR reflectance mode with slight overexposure. Signals beyond the dynamic range of the detector were highlighted as red pixels by the software. This feature assisted the user in detecting the areas of highest reflectivity and, therefore, the confocal plane with the largest overexposed area. Using this technique the dioptric focus was adjusted and the camera aligned so that the highest fundus NIR reflectivity was paracentral (uniform illumination in the entire 55° field of the mouse fundus is usually not possible using the Spectralis HRA). If the paracentral overexposure characteristic appeared similar in consecutive planes (sometimes spanning a range of up to 6 diopters [D]), the middle focus setting was chosen. Cross-sectional OCT imaging, using a wavelength similar to that of NIR reflectance, shows highest reflective bands at the approximate level of the RPE,⁴ suggesting that the highly reflective confocal NIR reflectance reference plane corresponds to anatomic structures at or close to the RPE.

The alignment for obtaining an optimal signal also ensured that the camera was aligned such that its "scan pupil" and its "detection pupil" were centered in the dilated pupil of the mouse and were in focus in the plane of the mouse's iris. The scan pupil is the area in the pupil plane in which the incident laser beam oscillates as it scans the fundus, and the detection pupil is the area through which the fundus AF is detected. Both pupils are conjugated to the scan mirrors in the camera and are concentric with each other. It is important to monitor the diameter of the dilated pupil during measurements because it may obstruct the scan pupil and the detection pupil and, thus, influence fundus AF measurements.

Images were usually recorded using the automatic real-time (ART) mode, which was able to track slight movements of the fundus (e.g., caused by respiration) based on fundus landmarks with high contrast. This allowed up to 100 consecutive frames to be averaged in real time and improved the signal-to-noise ratio. Single-averaged images were recorded with an intensity resolution of 8 bits/pixel, a 1536 × 1536 pixel resolution (high-resolution mode), and a frame rate of 4.8 frames/s. Rapid nonaveraged image sequences (high-speed mode, 768 × 768 pixel resolution, 8.9 frames/s) were used only for capturing photopigment bleaching effects.

For quantitative analysis, nonnormalized images were recorded in a dimmed room. Experiments on photopigment bleaching were performed in a dark room under dim red light after a minimum of 6 hours of dark adaptation. Normalized images (i.e., automatic software enhancement of contrast by histogram stretching) were only used if high contrast was aimed at (Fig. 10B). No further image processing was performed unless stated otherwise. The full angle of view using the 55° lens (Spectralis HRA) was displayed.

The detector sensitivity setting of the Spectralis HRA can be adjusted between 31 and 107, resulting in a nonlinear increase in detector gain by a factor of approximately 3600. Because detected AF levels in mice are low, a sensitivity of 100 was used to acquire all mouse images (except for the DsRed mouse, in which lower sensitivity settings are appropriate because of the expression of a fluorescent reporter protein). At this sensitivity, the zero-corrected GL (cGL) linear range extends up to approximately 150 GLs.²¹ In human subjects with healthy eyes, such sensitivity settings would usually result in nonlinear effects (i.e., GL > 150) and partial saturation, which is only rarely observed in mice. Although mouse fundus AF images may appear dark at a sensitivity ≤100, higher sensitivity settings were avoided because of an increasing contribution of noise and a decreasing linear range with increasing sensitivity.²¹

Quantitative Gray-Level Image Analysis

Quantitative GL analysis of nonnormalized and unprocessed fundus AF images (recorded with the same detector sensitivity) was performed using ImageJ software (developed by Wayne Rasband, National Insti-

FIGURE 1. Quantitative gray level (GL) analysis of fundus autofluorescence (AF) images. The *dashed line* in the graph (*bottom*) represents the GL profile along the horizontal 30 pixels wide band (*top*). The *continuous line* in the graph represents the corrected GL (cGL), which results after subtracting the zero-GL and which is used for all comparisons. The mean cGL within an annular area, concentric to the optic disc center, defined by *circles* with 250 and 450 pixels radius (*gray background, bottom graph*), was used for all quantitative GL measurements (indicated in the *left upper corner*; range 0–255 minus zero-GL). This sampling area avoids the optic disc and the more peripheral part of the image, where darkening and distortion are usually observed.

tutes of Health, Bethesda, MD; available at <http://rsb.info.nih.gov/ij/index.html>).²² After applying a standard Gaussian blur with a 3-pixel radius (σ) to reduce image noise, analysis included a plot of the mean GL profile within a 30-pixel-wide horizontal band through the optic nerve head and a measurement of the mean GL within the midperipheral fundus (Fig. 1). All measured GLs were corrected for the zero-GL, which is automatically recorded for individual images (“Grey value offset (0-level)” in the Image Information panel). This zero-signal is measured with the laser switched off but the detector on and thus is potentially affected by room light (dimmed room light was used for experiments). The resultant corrected GL (cGL) represented mean fundus AF and allowed compar-

isons between animals on the assumption that detector gain and laser diode power were both relatively constant and did not vary significantly in the ~12-month duration of this study.

Recording of the Pupil Diameter

The dilated pupil diameter in mice was usually larger than the diameter of the scan pupil, resulting in no relevant loss of the incident excitation light. However, the dilated pupil diameter was generally smaller than the diameter of the detection pupil, causing a loss of AF detection. To monitor this we measured the pupil diameter from an image of the pupil captured before each fundus AF image or each sequence of images. Each time, the focus was set at +50D, and the pupil was brought into focus by adjusting the z -position of the camera, with the eye in the center of the image to avoid distortions. This approach standardized the distance between camera and pupil plane and, hence, the magnification factor. The pupil diameter was measured in pixels using ImageJ after fitting a circular area to the largest pupil diameter. A scale factor of 83 pixels/1 mm, based on measurements of the image of the diameter of the contact lens centered in the iris image, allowed conversion to millimeters. The refraction at the anterior segment of the mouse eye, and especially at the interface air/contact lens, resulted in a magnification of the image of the pupil plane, acquired at a refraction setting of +50 D. This magnification was estimated by Zemax simulation to be in the order of 16% (Dr. Jörg Fischer, Heidelberg Engineering, data on file) and may explain differences of our measurements compared with smaller postmortem measurements of the pupil.²³ This magnification factor also affected the optical path. Thus, the calculated sizes of the scan pupil and the detection pupil (1.7 mm and 3.4 mm, respectively, for the 55° field lens; HRA2 55° Objective User Information, Heidelberg Engineering) were reduced to approximately 1.4 mm and 2.9 mm, respectively.

RESULTS

Image Acquisition

Contact Lens. Anesthetized mice with dilated pupils may develop cataract within few minutes, which may be related to corneal desiccation.²⁴ To investigate the optimal strategy to maintain clear ocular media throughout, fundus AF images were recorded using a contact lens (with Hypromellose coupling fluid as described earlier), artificial tears (Hypromellose) alone, or with neither of these protective measures (Fig. 2). The lack of corneal protection consistently resulted in cataract formation and reduced fundus AF signal intensity in minutes. Contact lens placement was found to be a reliable means to avoid this. Application of artificial tears alone also resulted in a protective effect, though this was more variable than with the contact lens.

FIGURE 2. Prevention of media opacity using a contact lens. Cataract development in anesthetized mice with dilated pupils, without measures to prevent corneal desiccation, results in image deterioration within minutes (*upper row*). Quantification of the decreasing fundus AF (normalized to the first measurement at 0 minutes) is shown in the graph (*triangles*). Such media opacity can be avoided by protecting the cornea from drying using a contact lens (*lower row, horizontal bars*). A similar but less consistent effect can be achieved using a corneal lubricant (*circles*). cGL, corrected gray level.

FIGURE 3. Correct placement of the contact lens (CL) is necessary for recording images with even illumination of the fundus (*upper row*). Displacement of the contact lens results in shadowing of the fundus image (*lower row*; arrows mark lens margin). Adequate lens position can be controlled using the cSLO image focused on the anterior segment (e.g., +50 D).

We found that contact lens use allowed better comparability between animals because of the consistent curvature of the contact lens. Additionally, the small eyes of very young mice could be imaged with the contact lens application. The contact lens may lead to a change in magnification and thus a more or less wider fundus area displayed.

For optimal recordings of the posterior pole, the contact lens had to be placed exactly on the corneal center (Fig. 3). Off-center lens position caused shadowing in the direction of lens displacement. However, when imaging the peripheral retina, deliberate displacement of the lens toward the incident light beam usually improved peripheral fundus illumination.

Camera Position. Uneven retinal illumination may also be caused by poor camera alignment with the pupillary axis and plane and improper camera position along the *z*-axis (Fig. 4, upper row). Notably, the latter factor also significantly affected image magnification. Translation of camera *z*-position by only 4 mm resulted in an approximate doubling of the apparent optic disc diameter (Fig. 4, bottom row).

Focus. After aligning the camera using the NIR reflectance mode (820 nm laser), a dioptric shift of approximately +8 D to +10 D (due to chromatic aberration) is needed to refocus the

FIGURE 5. Dioptric shift with change in illumination wavelength. When using the 820 nm reflectance image for positioning of the camera and locating the confocal plane of interest (*upper left*), the 488 nm fundus AF image is not in focus (*upper right*; the vascular walls are visible because of expression of the fluorescent reporter protein DsRed under control of the actin promoter). The confocal plane must be corrected by approximately 8 D. Images are cropped from images recorded with the 55° field lens.

488 nm fundus AF image on the same retinal plane as the NIR image (Fig. 5). Focusing on other layers of the retina then necessitates additional dioptric shifts. The shorter focal length of the mouse eye results in greater confocality than in humans. Thus, a large change of approximately +30 D to +40 D was necessary to change the focus from the outer retinal reference plane (see Methods) to the nerve fiber layer of the inner retina in adult wild-type mice. With the contact lens in place and maximal pupil dilation, a focus setting of about +30 D was often a good estimated initial configuration to identify the outer retinal reference plane in adult mice. However, mouse strain, age, and pupil dilation may considerably affect the nature of the dioptric adjustments necessary for optimal focus.

Pupil Diameter. The pupil size in mice after dilation with tropicamide and phenylephrine varies greatly with age (exem-

FIGURE 4. Influence of camera position on fundus shadowing and magnification. Misalignment from an optimized camera position results in peripheral image shadowing (*upper row*). Changes of the camera position in the *z*-axis led to significant changes in image magnification and thus influenced the scale factor (*bottom row*). The focus was readjusted for each camera position. A degenerated retina was recorded using the NIR reflectance mode because of the superior visibility of the disc margin.

FIGURE 6. (A) Pupil size increases with age, especially during the first 3 months of life. (B) Effect of different pupil diameters on AF measurement, exemplified in a 5-month-old *Abca4*^{-/-} mouse. The midperipheral cGL was measured (indicated in the upper left corner of each fundus image). Insets: NIR iris images recorded immediately after the AF image. (C) Log-log plots showing the variation of fundus AF with pupil diameter for five eyes (different symbols, minimum of four measures per eye). The curves were slightly displaced vertically for clarity ($\leq 5\%$). Interrupted line: theoretical prediction of how AF would change with change in pupil diameter in the assumptions those pupils are concentric with the iris, that the laser irradiance in the scan pupil is uniform, and that the pupil diameter is recorded at the same time as the fundus AF image. Arrows: diameters of the scan pupil (S) and the detection pupil (D), respectively. Black horizontal bar: range of pupil diameters for all other instances in this study.

plified for C57Bl/6 mice in Fig. 6A). The dilated pupil diameters measured in this study ranged from 1.9 mm to 2.3 mm (Fig. 6C, black bar) and thus remained below the diameter of the detection pupil. To assess the effect of pupil size on AF measurement, repetitive combined measurements of pupil size and fundus AF intensity were obtained during successive pharmacologic pupil dilation (phenylephrine in fellow eye, tropicamide in recorded eye, additional phenylephrine in recorded eye). The focus of the reference plane (see Methods) changed slightly for each pupil diameter (most likely because of spherical aberrations) and thus was adjusted for each fundus AF image. Figure 6B illustrates the effect of increasing pupil size in the same eye on fundus AF. Measurements in our study were at pupil diameters between that of the scan pupil and that of the detection pupil (Fig. 6C). The time needed to refocus and reposition the camera between fundus and pupil imaging, and the relatively fast pupil dilation in mice, remained sources of imprecision in our study when attempting to correlate pupil diameter with fundus AF measurements.

Recording and Quantifying Autofluorescence from the RPE

cGL analysis of fundus AF images from the reference plane (see Methods) and adjacent confocal planes suggested that the reference plane was a good approximate position from which to record the highest AF signal originating from the RPE (Fig. 7A). The deviation of the mean cGL from the mean maximum cGL was $\leq 5\%$ within ± 4 D defocus and $\leq 10\%$ within ± 8 D defocus from the reference plane. The highest fundus AF signal was always measured within ± 6 D defocus from the reference plane. More severe defocus further reduced the AF signal detected (Fig. 7A).

The test-retest variability of RPE AF measurements was assessed in 31 eyes of 31 animals (C57Bl/6 and *Abca4*^{-/-}). After the reference plane was identified, a 488 nm fundus AF image was recorded (test 1). The mouse was then taken off the platform, and the contact lens was removed. Hypromellose eyedrops were applied again, the contact lens was replaced

FIGURE 7. Recording AF from the RPE in mice. (A) Effect of defocus from a reference plane, defined as the confocal plane with the highest reflectivity in the NIR reflectance mode. AF images were recorded in 2 D steps in a range of ± 10 D defocus from the reference plane and 5 D steps outside this range up to ± 25 D defocus; $n = 13$. Mean $\pm$ SD. (B) Bland-Altman plot of percentage difference against mean for quantitative fundus AF measurements (cGL). Continuous line: mean difference; dashed lines: 95% limits of agreement.

FIGURE 8. Effect of blue light exposure on the measured fundus AF signal. (A) Photopigment bleaching increases fundus AF considerably within the first 1.5 seconds of blue light exposure in wild-type C57Bl/6 mice. This increase is not seen in *Rho*^{-/-} mice that lack photopigment. The mean $\pm$ SEM of four independent measurements is shown. (insets) Enhanced fundus differential maps (at 2 seconds minus baseline) in wild-type (top) and *Rho*^{-/-} mice (bottom). (B) During continuous light exposure over 3 minutes (lower horizontal white bar), there is an approximately 15% decrease in measured fundus AF (circles, solid line). A similar change was observed after a 3-minute interval without light exposure, during which AF remained stable (upper black and white horizontal bar). The mean $\pm$ SEM of four independent measurements in C57Bl/6 mice is shown.

and imaging was performed (test 2) as in test 1. Midperipheral cGL was measured as described earlier in the Methods. Measurements from test 1 and test 2 were not significantly different ($P = 0.93$, paired t -test). The intersession coefficient of repeatability ($1.96 \times$ SD of the differences between the two measurements) was approximately $\pm 22\%$ (95% confidence interval; Fig. 7B).

Bleaching Experiments

Bleaching of rhodopsin in photoreceptors changes its absorption maximum ($\lambda_{\max}$) from ~ 500 nm to ~ 330 nm, which leads to decreased 488 nm light absorption by the photoreceptor layer. This results in higher 488 nm light exposure of the RPE layer and thus an increased excitation of RPE AF.^{21,25}

Because of the high retinal irradiance (~ 30 times what is used in human with the 30° lens; see Discussion) and no possibility to reduce the excitation light through the user interface, we predicted that bleaching would occur much more rapidly than the ~ 30 seconds it takes in humans.²¹ Therefore, the “high-speed” movie mode was started and the laser activated while the optical path was manually occluded with black card. Rapid removal of the occluder allowed immediate high speed image sequence acquisition from the moment of 488 nm laser bleaching onset. There was an $\sim 40\%$ increase in fundus AF in the first 1.5 seconds of laser illumination in 2-month-old C57Bl/6 mice that leveled off thereafter (Fig. 8A). In age-matched *Rho*^{-/-} mice, in which rhodopsin as the major photopigment in the retina is absent, no change in fundus AF intensity was noted. Photopigment bleaching and thus photopigment distribution can be mapped topographically by subtraction of unbleached from a bleached fundus AF image (Fig. 8A).

In addition to photopigment bleaching, we observed a slow decrease of fundus AF during several minutes of blue light exposure. Continuous exposure to the excitation light over 3 minutes resulted in lower fundus AF measurements. If a 3-minute control interval preceded the continuous light exposure, there was no change during the initial dark phase.

Fundus Autofluorescence Imaging in Wild-Type Mice and Disease Models

Figure 9 compares 488 nm and 790 nm fundus AF pattern and intensity in three mouse strains (WT129, C57Bl/6,

BALB/c) commonly used in retinal research and in the *Abca4*^{-/-} mouse, a model of Stargardt disease. Patients with Stargardt disease have increased 488 nm fundus AF⁸ because of excessive accumulation of lipofuscin in the RPE.

Fundus AF at 488 nm was lowest in the two wild-type strains, WT129 and C57Bl/6. In BALB/c mice, the dots of increased AF may represent subretinal photoreceptor debris³ or lipofuscin-containing macrophages and/or microglia cells, as described in another mouse model with a similar phenotype.²⁶ Notably, the resultant jagged gray level profile shows a higher background signal compared with the two wild-type strains, possibly because of the increased accumulation of lipofuscin fluorophores or, alternatively, the increased excitation light from the lack of iris and fundus pigmentation. The cGL in the *Abca4*^{-/-} mouse was approximately twice the level in the wild-type strains. There was a pattern of focally increased AF in the *Abca4*^{-/-} mouse, revealing a faint flecked phenotype similar to the flecked fundus AF appearance in Stargardt patients.

AF at 790 nm is thought to originate from melanin in the RPE and choroid. Accordingly, the AF signal was almost absent in the nonpigmented BALB/c mouse when compared with pigmented mouse strains. Fundus AF 790 nm intensity in the *Abca4*^{-/-} mouse on a WT129 background was about twice as high as in WT129 mice.

Figure 10 shows fundus AF images from two mouse models of retinal dystrophies. *Pde6b*^{rd1/rd1} mice homozygous for the *rd1* mutation have an early-onset, fast retinal degeneration with loss of all rods within the first month of life.²⁷ *Rho*^{-/-} mice have an absence of rod function caused by the lack of rhodopsin. At 3 months of age, photoreceptor degeneration is essentially complete.¹⁹

Both animal models show a marked reduction in 488 nm fundus AF intensity at the age of 3 months compared with wild-type animals (Fig. 10A). This finding is consistent with a lack of lipofuscin accumulation from the absence of a normal visual cycle. Fundus AF at 790 nm was lower in the *Pde6b*^{rd1/rd1} mouse than in the wild-type mouse but was slightly higher in the *Rho*^{-/-} mouse. Only after normalization by histogram stretching did both imaging modalities reveal an altered pattern

FIGURE 9. Pattern and corrected gray level (cGL; representing fundus AF intensity) on 488 nm and 790 nm fundus AF images in three wild-type mouse strains and an *Abca4*^{-/-} mouse at 6 months of age. Standardized image acquisition and detector settings allow quantification and comparison of the AF intensity. The numbers in the left upper corner of each fundus image represent the mean cGL measured in the midperiphery. The horizontal cGL profile through the optic disc is shown in the bottom line plots for 488 nm (left) and 790 nm (right) fundus AF images. Pupil diameter was 2.2, 2.33, and 2.26 mm in the WT129, C57Bl/6, and *Abca4*^{-/-} mice, respectively, and was not recorded in the BALB/c mouse because of the translucent iris.

FIGURE 10. GL (A) and pattern (B, contrast enhanced) fundus AF images in two mouse models for retinal dystrophies. (A) Both models have a lack of photoreceptor outer segment shedding and thus do not accumulate lipofuscin, resulting in a reduced AF compared with an age-matched wild-type mouse. Pupil diameter was 2.23, 2.28, and 2.30 mm in the C57Bl/6, *Rho*^{-/-}, and *Pde6b*^{rd1/rd1} mouse, respectively. cGL, corrected gray level. (B) Histogram stretching and contrast enhancement reveals a pattern of further decreased AF that first appears on 790 nm AF images. All alterations occur earlier in the faster progressing *Pde6b*^{rd1/rd1} model. *Rho*^{-/-}, rhodopsin knockout mouse. *Pde6b*^{rd1/rd1}, nonsense mutation in *Pde6b*.

compared with normal (Fig. 10B). The slow progressive degeneration in the *Rbo*^{-/-} mouse revealed a multitude of fluorescent spots on 488 nm AF, possibly representing activated microglia,²⁸ which appeared to increase in number with age. At 7 months, large patches with a decreased signal were visible only on 790 nm AF. In a 16-month-old *Rbo*^{-/-} mouse, these areas were also faintly visible on 488 nm fundus AF images. In the *Pde6b*^{rd1/rd1} mouse, onset of similar phenotypic observations occurred at earlier time points; at 2 months there were areas of reduced 790 nm AF signal that subsequently became apparent on 488 nm AF. *Pde6b*^{rd1/rd1} mice demonstrated higher AF signal intensity (relative to adjacent areas) at the border of atrophic areas, which was not seen in *Rbo*^{-/-} mice.

DISCUSSION

In this study, the parameters affecting fundus AF imaging in mice were studied so the procedure could be optimized and standardized to allow quantitative assessment of FA. A standard protocol for fundus AF imaging in mice has been designed (Supplementary Material, <http://www.iovs.org/lookup/suppl/doi:10.1167/iovs.11-8767/-/DCSupplemental>) and was subsequently applied to mouse models for retinal diseases.

Image Acquisition

Considerable improvement in image quality may be achieved by using a contact lens (Fig. 2). Media opacities decrease the detectable signal and the signal-to-noise ratio of the already low fundus AF signal in mice. The contact lens prevents the development of cataract and ensures a consistent surface profile across different mice. It also enables recording from very small eyes in young animals in which adjustment of the confocal plane can otherwise be very difficult.

A scale factor would allow the conversion of cSLO fundus image measurements in pixels into actual size. Differences in the optical properties and sizes of the human and mouse eyes render current software algorithms unsuitable for use in mice. Assuming a full angle of view of the murine fundus with the 55° lens, a 1.1- to 1.2- $\mu\text{m}/\text{pixel}$ scale factor may be calculated based on the number of pixels per degree (1536 pixels per 55° fundus image = 28 pixels/1° visual angle) and estimates of the lateral measure of the visual angle on the mouse retina (1° visual angle = 31 to 34 μm ^{23,29}). The exposed circular retinal area can thus be calculated as $3.14 \times (1.1\text{ to }1.2\text{-}\mu\text{m}/\text{pixel} \times 768\text{ pixels} \times 10^{-3}\text{ mm}/\mu\text{m})^2$ or 2.2 to 2.7 mm^2 .

We observed that small changes in camera z-position resulted in large effects on image magnification (Fig. 4, bottom), a finding not usually obvious in humans. Tests with graph paper (not shown) revealed that this effect increases with high positive focus settings as used in mice, which are rarely needed in patients. The dependency of magnification on the distance between the retina and the camera, and thus on eye growth, implies that fixed scales for cSLO images of the mouse fundus will remain imprecise. Possible effects of image magnification on AF measurements were therefore not accounted for in this study.

Both the dioptric shift necessary to correct for chromatic aberrations (Fig. 5) and the high confocality of the mouse eye are in line with previous reports,^{2,30} with the latter allowing for excellent discrimination between retinal planes. Retinal thickness measurements in vivo revealed variable results in mice, ranging from $\sim 200\text{ }\mu\text{m}$ to $\sim 300\text{ }\mu\text{m}$.^{31,32} Based on the +30 to 40 D change necessary to focus from the outer to the inner retinal border, an effect of ~ 5 to $10\text{ }\mu\text{m}$ change per diopter change in focus may be estimated. A lower dioptric range between inner and outer retina may suggest retinal atrophy.

Recording and Quantifying Autofluorescence from RPE

Fundus AF originates mainly from fluorophores in the RPE⁶ and therefore from a defined plane between Bruch's membrane and the neurosensory retina. Reliable localization of this confocal plane while recording fundus AF is difficult to achieve in mice because of the lack of fluorescent structures or landmarks with high contrast or signal intensity, and only minor effects on measured AF with small focus changes in the outer retinas of mice (Fig. 7A). A reference plane of highest NIR reflectivity may instead be used to approximate the plane of highest fundus AF (Fig. 7A). An origin of the high NIR reflectance from the Bruch's membrane/RPE complex is supported by human cSLO reflectance studies that suggest higher reflectivity caused by melanin³³ and by the high reflectivity from the Bruch's membrane/RPE complex on OCT recordings using a similar wavelength in the NIR range. Small dioptric shifts between highest AF signal and reference plane may be attributed to slightly different planes of origin, chromatic aberrations, and minor measurement errors. The only small changes of measured AF with defocusing ± 4 D from the reference plane may be explained by the high confocality in the mouse eye, resulting in a change of only ± 20 to $40\text{ }\mu\text{m}$ across the RPE.

Measurement of fundus AF in mice is substantially affected by the small size of the dilated pupil in mice, restricting the detection pupil and thus underestimating the fundus AF (Fig. 6). The scan pupil was generally smaller than the dilated pupil. When pupil diameter was varied in the same animal, we found a near-exponential relationship between fundus zero-cGL and pupil diameter (log-log plot; Fig. 6C). The slopes for the five animals varied between 1.4 and 2.8 (mean, 2.0 ± 0.4), indicating that the cGL was roughly proportional to the area of the pupil aperture (the data for the largest diameter were removed because these might have been equal to or larger than the diameter of the detection pupil). This suggests that correction for loss of AF because of pupil obstruction may be feasible, but further experiments are needed to confirm these somewhat variable data. Smaller custom-made cSLO apertures may be used in future studies to reduce both the size of the scan pupil and the detection pupil, though at the expense of resolution and signal strength.

When measuring fundus AF in mice, the procedure as performed in this study has limitations. To allow comparison between mice with different pupil diameters (e.g., in longitudinal observations and when comparing fundus AF between mice of different ages), a correction for variation in pupil diameter would be needed. Such correction was not implemented in this study because pupil diameters were about equal when AF measurements were compared (Figs. 9, 10). Furthermore, variability between measurements could be minimized by using an internal reference, as described recently in a human study.²¹ It would account for short-term (warm-up) and long-term changes in laser power and detector sensitivity fluctuations and would provide a check for the consistent functioning of the camera. An internal reference would also permit expression of fundus AF measurements in a format that can be compared among studies obtained with different cameras. However, variation in fundus AF measurements between operators and because of suboptimal standardization of the recording procedure (e.g., differences in pupil dilation, camera alignment, control of media opacity) appear to have a higher impact on fundus AF measurements in mice than in humans. Thus, an internal reference would increase confidence on the functioning of the setup, but variability is likely to remain higher than in humans.

Bleaching Experiments

An effect of photopigment bleaching on fundus AF in mice was observed within the first 1.5 seconds of exposure to the excitation light using the 55° cSLO lens (Fig. 8A), which is much faster than in human AF imaging using the 30° cSLO lens (20–30 seconds^{21,25}). The difference can be explained by the fact that approximately the same beam power ($\sim 260 \mu\text{W}$) is used to scan a smaller retinal area in mouse (2.2–2.7 mm²) than in human imaging (79 mm², with a 30° field). As a result, the retinal irradiance occurring in mouse imaging is approximately 30 times higher than in human imaging (488 nm AF imaging using the Spectralis HRA; $\sim 10 \text{ mW/cm}^2$ and 0.3 mW/cm^2 for mice and humans, respectively, ignoring media-related light loss).

In addition, a slight decrease of fundus AF over time was observed during continuing blue light exposure. A photochemical effect of relevant fluorophores or a masking of fluorophores by photochemical generation of additional absorbers might explain this finding. A reversible decrease in fundus AF (“fading”) has been observed in monkeys after exposure to 5 to 210 J/cm² with 568 nm light over long exposure periods (15 minutes).^{34,35} The lower end of the range observed in monkeys would just approximate the 1.8 J/cm² retinal radiant exposure during 488 nm cSLO AF imaging in mice (retinal irradiance of 10 mW/cm² for 3 minutes). More experimental evidence is needed here to ascertain whether this represents the same process.

Photoreversal of bleaching is a process by which rhodopsin is photochemically regenerated within photoreceptors during intense blue light exposure. The reported kinetics appears to be in line with our experiments. The effect was observed in rats with a retinal irradiance of 300 $\mu\text{W/cm}^2$ using 403 nm light,³⁶ which is ~ 2 log units below the irradiance during 488 nm AF imaging (Spectralis HRA) in mice. If photoreversal of bleaching underlies the observed decrease in fundus AF, the higher irradiance might compensate for a mismatch of the 488 nm excitation light with the absorption maximum of the suggested photoreversible intermediate.

Fundus Autofluorescence Imaging in Different Mouse Strains and Models of Retinal Disease

Light at 488 nm is close to the excitation maximum of lipofuscin,⁶ of which major components accumulate extensively in the *Abca4*^{-/-} mouse.¹⁸ Consistent with this, we observed 488 nm AF to be much higher in the *Abca4*^{-/-} mouse than in wild-type animals (Fig. 10). Additionally, mice with retinal degenerations, in which a lack of visual cycle byproducts would be expected to result in less A2E accumulation (*Pde6b*^{rd1/rd1} and *Rho*^{-/-} mouse), had a much lower fundus AF (Fig. 10A). Such quantitative AF measurements may be useful in humans to classify retinal dystrophies according to their AF levels. This may suggest certain genetic defects or pathophysiological pathways, as exemplified previously for patients with mutations in *ABCA4*, *RPE65*, *RDH5*, or *MERTK*.^{8–11} Furthermore, excitation at 790 nm, which has been suggested to excite melanin fluorescence,^{12–14} results in a higher fluorescence signal in highly pigmented mice and only very low signals in albino mice (BALB/c). Interestingly, the *Abca4*^{-/-} mouse showed the highest 790 nm fundus AF. Longitudinal and histopathologic studies may shed light on the nature of this finding, which is in line with human data that have thus far remained unexplained.¹⁵

Histogram stretching and contrast enhancement of AF images in *Rho*^{-/-} and *Pde6b*^{rd1/rd1} mice produced images reminiscent of human retinal dystrophies (Fig. 10B). In early stages, there was a pattern of spotted relatively increased AF. Histologic studies suggest that such foci of increased AF may corre-

late with lipofuscin-containing macrophages, microglia cells, or both.^{26,28,37} A progressive localized loss of fundus AF develops over time. The faster progressing phenotype (*Pde6b*^{rd1/rd1} mouse) revealed areas of increased AF surrounding areas of presumed atrophy, which was not observed in the slowly progressing phenotype (*Rho*^{-/-} mouse). Similar phenomena have been described in patients, in whom increased AF surrounding atrophy may be a marker for disease progression.^{5,38} Notably, in the *Rho*^{-/-} and the *Pde6b*^{rd1/rd1} mouse, only contrast enhancement made these patterns visible against a very low fluorescent background. Thus, such patterns are not necessarily indicative of abnormal and potentially cytotoxic lipofuscin accumulation but might rather reflect multilayered RPE cells, photooxidized fluorophores, or impaired photoreceptors.³⁹ Moreover, changes appear to be visible earlier on 790 nm AF, which is in line with findings in patients in whom 790 nm fundus AF often shows larger affected areas than other imaging modalities, including 488 nm fundus AF.^{16,17}

CONCLUSION

We have shown that consistent and reproducible fundus AF imaging is feasible in mice through adherence to key principles such as the prevention of media opacities and standardization camera position and confocal plane during image acquisition. Fundus AF, now increasingly accepted as a meaningful clinical outcome measure, may allow noninvasive and longitudinal assessment in preclinical models, potentially reducing the number of research animals needed. Furthermore, phenotypic similarities with fundus AF findings in patients suggest that animal models for retinal diseases may be used to investigate further the correlation between histopathology and disease-related AF phenomena. Our findings support the clinical use of AF imaging in the definition of different phenotypes of retinal degeneration in monitoring disease progression and the possible response to innovative treatments.

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