Interpretations of Fundus Autofluorescence from Studies of the Bisretinoids of the Retina

Janet R. Sparrow,^{1,2} Kee Dong Yoon,¹ Yalin Wu,¹ and Kazunori Yamamoto¹

Monitoring of monogenic and multifactorial forms of retinal degeneration, including age-related macular degeneration (AMD) and some forms of retinitis pigmentosa (RP), often includes fundus autofluorescence imaging, a modality that primarily relies on the fluorescence generated from the bisretinoids of lipofuscin in retinal pigment epithelial (RPE) cells.^{1–3} These bisretinoids form initially in photoreceptor cells and are deposited secondarily in the RPE.

In fundus autofluorescence images, geographic atrophy (GA), and even smaller isolated patches of atrophy, can be recognized as areas of profoundly reduced fluorescence.^{4,5} Often noticed but less understood are elevated autofluorescence signals, including the high-intensity autofluorescence observed at the margin of GA⁴ and within the parafoveal rings of intense autofluorescence that occur in some patients with RP.⁵ In this article, we visit factors that can contribute to fundus hyperautofluorescence and we provide evidence that the amount of lipofuscin in RPE cells is not the only factor governing fundus autofluorescence intensity. Photooxidation of RPE lipofuscin can also result in heightened fluorescence emission. In addition, we propose that impaired handling of vitamin A aldehyde in the setting of RPE and photoreceptor cell dysfunction and atrophy can result in excessive production of bisretinoid fluorophores in photoreceptor cells, such that these cells become an anomalous yet major source of fundus autofluorescence. The relevance of these issues to fundus autofluorescence in AMD and monogenic retinal disorders is discussed.

CHARACTERISTICS OF FUNDUS AUTOFLUORESCENCE

The fundus of the retina exhibits an intrinsic fluorescence that has been quantified by noninvasive spectrofluorometry⁶ and imaged with confocal scanning laser ophthalmoscopy (cSLO)² (Fig. 1), a fundus camera-based system,⁷ and by adaptive optics.⁸ The spectral characteristics, spatial distribution, and age-dependent intensities of fundus autofluorescence are consistent with the source of this natural autofluorescence being the lipofuscin pigments that accumulate inside retinal pigment epithelial (RPE) cells.^{1,3} Moreover, observations of fundus autofluorescence in relation to macular pigment, macular

holes, and RPE atrophy indicate that fundus autofluorescence originates from RPE lipofuscin. Mapping of the topographic distribution of fundus autofluorescence has revealed that autofluorescence is more pronounced superotemporally than inferonasally, and eccentric levels are greater than at the fovea.^{9,10} In individuals with normal retinal status, fundus autofluorescence increases linearly with age, although there is pronounced variation among subjects.¹⁰ The leveling off of this increase after age 70 could reflect undercorrection of lens absorption,¹¹ a loss of RPE cells,¹² and/or shifts in the excitation maxima caused by extensive photooxidation of the bisretinoid compounds.¹³

The autofluorescence of the fundus can be excited across a broad range of wavelengths; the excitation used for imaging with a cSLO is typically 488 nm; for imaging by fundus camera, 535 to 580 nm⁷; and for spectrofluorometry, 550 nm.¹⁰ The fluorescence emission of fundus autofluorescence is broad and centered at approximately 610 nm.^{1,10} This emission is markedly similar to the emission spectra of native lipofuscin present in RPE harvested from human eyes, and in both cases the emission exhibits a red shift with increasing excitation wavelength.¹⁴

FLUORESCENT BISRETINOID AS THE SOURCE OF FUNDUS AUTOFLUORESCENCE

A Complex Composition

Despite speculation that RPE lipofuscin is protein-based, amino acid analysis of purified lipofuscin granules, the lysosomal bodies in which RPE lipofuscin is housed, has revealed only 2% amino acid content.¹⁵ Instead, all the fluorescent constituents of RPE lipofuscin identified to date are bisretinoid compounds that form in photoreceptor cells from random inadvertent reactions of vitamin A aldehyde (all-*trans*-retinal), the latter being produced on photon absorption by the visual pigment chromophore 11-*cis* retinal. The bisretinoid compounds that comprise the lipofuscin material are deposited in RPE cells secondarily. This pathway of lipofuscin synthesis explains the absence of fundus autofluorescence in patients with early-onset retinal dystrophy associated with mutations in RPE65¹⁶; the latter protein is the visual cycle isomerase essential to the formation of the visual pigment chromophore 11-*cis*-retinal. For all these bisretinoid chromophores, the fluorescence emission is broad and peaks at approximately 610 nm; however, these pigments exhibit various excitation maxima in the visible spectrum, including 430 nm (all-*trans*-retinal dimer), 439 nm (A2E), 426 nm (iso-A2E), 490 nm (A2-dihydropyridine-phosphatylethanolamine, A2-DHP-PE), and 510 nm (all-*trans*-retinal dimer-phosphatidylethanolamine and all-*trans*-retinal dimer-ethanolamine).^{17–20} This range of excitation wavelengths could affect fundus autofluorescence imaging. Depending on whether one is imaging with a cSLO and 488 nm excitation or with a modified fundus camera and longer wavelengths (535–580 nm excitation), contributions to the images

From the Departments of ¹Ophthalmology and ²Pathology and Cell Biology, Columbia University, New York, New York.

Supported by National Institutes of Health Grant EY 12951, the Kaplen Foundation, a grant from Research to Prevent Blindness to the Department of Ophthalmology, and a postdoctoral fellowship from National Research Foundation of Korea (KY).

Submitted for publication May 10, 2010; revised June 11, 17, and 17; accepted June 18, 2010.

Disclosure: **J.R. Sparrow**, None; **K.D. Yoon**, None; **Y. Wu**, None; **K. Yamamoto**, None

Corresponding author: Janet R. Sparrow, Columbia University, Department of Ophthalmology, 630 W. 168th Street, New York, NY 10032; jrs88@columbia.edu.

FIGURE 1. (A) Fundus autofluorescence image obtained from an adult with healthy retinal status: cSLO and 488 nm excitation. (B) Fundus images obtained from an individual with GA: autofluorescence (left) and color fundus (right) photographs. A zone of increased autofluorescence signal (arrows) surrounds an irregular and nonhomogeneous zone of reduced AF, with uniform loss of AF occurring most centrally.

from different subgroups of the lipofuscin fluorophores could vary.

The immediate precursor of A2E is the phosphatidyl-pyridinium bisretinoid A2PE (excitation maximum, ~ 449 nm). This pigment forms in photoreceptor cell outer segments from reactions of all-*trans*-retinal with the phospholipid phosphatidylethanolamine.^{17,21–23} Enzyme-mediated phosphate hydrolysis of A2PE in RPE cells removes phosphatidic acid and releases A2E (for further discussion of the relationship between A2PE and A2E, see the legend to Fig. 4). In a healthy retina, A2PE and other compounds of the lipofuscin pathway are kept to a minimum in photoreceptor cells by means of daily shedding of outer segment membrane followed by RPE-mediated phagocytic clearance.²⁴ As a result of this process of membrane renewal, the entire photoreceptor outer segment is turned over every 10 to 14 days, and the vitamin A aldehyde adducts that will become the lipofuscin are deposited into the lysosomal compartment of the RPE where they appear to be refractory to enzyme degradation. Thus, at any given time, the lipofuscin in RPE consists of at least some portion of the fluorescent material that has accumulated since birth, whereas the lipofuscin-associated pigment in outer segments has formed more recently.

Conditions Influencing Bisretinoid Formation

The compounds that will become the lipofuscin of RPE form in the membranes of photoreceptor outer segments before phagocytosis of those same membrane segments by the RPE. The formation of these bisretinoid lipofuscin fluorophores is light dependent. For instance, A2PE, the immediate precursor of A2E, is augmented in rats exposed to bright light,²² but its production is arrested in dark-reared mice.²⁵ The efficiency with which the retinoid cycle replenishes the 11-*cis* chromophore of cone and rod visual pigment determines all-*trans*-retinal flux and thus is tightly coupled to the formation of lipofuscin bisretinoids.^{26–30} Even more so, conditions that interfere with clearance of all-*trans*-retinal from the interior of outer segment discs result in accelerated formation of the bisretinoids. For instance, the activity of ABCA4 (ABCR), the photoreceptor-specific ATP-binding cassette transporter,^{31–34} is a determinant of all-*trans*-retinal availability. Mutations in *ABCA4* are responsible for recessive Stargardt disease,³⁵ and some forms of cone-rod dystrophy and RP.³⁶ As a consequence of deficient ABCA4-facilitated removal of all-*trans*-retinal from the interior of outer segment discs, RPE lipofuscin is elevated in ABCA4-related disease and in *Abca4*^{−/−} mice.^{27,37–40} Knockout of the photoreceptor cell enzymes (all-*trans*-retinol dehydrogenases) responsible for detoxifying all-*trans*-retinal (by conversion to all-*trans*-retinol) also leads to

enhanced formation of bisretinoid.^{30,41} Of course there may be other as yet unidentified factors that enhance or restrain bisretinoid formation. A widely held assumption is that the pace of formation of RPE lipofuscin is dependent on the rate of phagocytosis of outer segment membrane. However, as discussed earlier, the bisretinoids of lipofuscin are assembled in the photoreceptor cell before disc shedding and phagocytosis; thus, although their production can be modulated by factors such as light and the efficiency with which reactive all-*trans*-retinal is managed, their generation would be expected to be independent of the rate at which outer segment membrane is shed and cleared.

FUNDUS AUTOFLUORESCENCE IN RETINAL DISORDERS

Fundus autofluorescence can exhibit topographic changes in intensity in the presence of retinal disease such as AMD. For instance, areas of markedly deficient or absent fundus autofluorescence signal are recognized as corresponding to regions of RPE and photoreceptor cell demise (e.g., GA)^{4,42} (Fig. 1B). Loss of the RPE cell monolayer in GA has been confirmed by OCT (optical coherence tomography).⁴³ These areas of atrophy have been observed to expand with time, although growth rates vary considerably among individuals.^{44–47}

Interest in correlating changes in fundus autofluorescence with retinal disease has led investigators to pay particular attention to the zone of retina surrounding GA (Fig. 1B). In fundus autofluorescence images this junctional area is often marked by elevated levels of brightness.^{3,45} With time, outward enlargement of GA occurs, but outward extension of the band of elevated autofluorescence always precedes growth of the area of absent autofluorescence (atrophy).^{4,48}

The rate of progression of atrophy has been reported to correlate with the pattern of hyperautofluorescence observed in the junctional zone.^{47,49,50} For example, a continuous zone of autofluorescence intensity encircling an area of atrophy portends higher rates of atrophy than does a surround of multiple intermittent foci of elevated brightness.^{47,49,50} Indeed, this risk-associated patterning in the junctional zone is more strongly associated with rates of atrophy progression than other factors such as smoking and age.^{47,49,50} Reduced light sensitivities, measured by fundus perimetry using either a modified Humphrey field analyzer (Carl Zeiss Meditec, Dublin, CA)⁵¹ or SLO,⁵² indicate impaired photoreceptor function in the area of increased autofluorescence surrounding GA in AMD. The precise significance of the increased autofluorescence signal at junctional zones of GA in AMD has been a topic of interest.

Abnormal fundus autofluorescence in the form of parafoveal rings of high intensity have also been reported in some individuals with RP. These rings of hyperautofluorescence are not genotype-specific,⁵³ and they demarcate central areas of preserved macular function. A gradual reduction in ring diameter reflects progressive visual field loss.^{5,54,55}

In contrast to cases of RP, *ABCA4*-related disease and cone-rod dystrophies of other origins can present with autofluorescent rings that surround decreased or absent foveal autofluorescence and that progressively expand with time.^{53,56} These rings co-localize with areas of reduced visual sensitivities,⁵ and across the annulus, inner segment-outer segment junctions may not be visible on SD-OCT images.⁵⁷

ACCOUNTING FOR ENHANCED AUTOFLUORESCENCE

Elevated Levels of Lipofuscin in RPE Cells

The fluorescence emission of bisretinoids of lipofuscin such as A2E and all-*trans*-retinal dimer increases with their abundance (Fig. 2); thus, there is no doubt that fluorescence intensity can reflect the magnitude of the accumulation in RPE cells. Most notably, these accumulations are particularly augmented in recessive Stargardt disease.^{39,58,59} However, it has been assumed that hyperautofluorescence at the edge of GA or in the perifoveal rings of some forms of monogenic retinal disease, are also indicative of exceptional levels of lipofuscin in the RPE.^{4,5,48,49,60–62} In the case of GA, the implication has been that an excess of lipofuscin in the RPE predisposes to the expansion of atrophy.⁴ However, one would have to identify a mechanism by which the RPE, in the limited zone surrounding

GA or in the perifoveal rings of RP, could acquire elevated levels of lipofuscin in a relatively short time. Focally apparent hyperautofluorescence originating from RPE could also be associated with displacement and clumping of the lipofuscin-laden cells^{4,45}; in those cases, OCT could be confirmatory.

Hyperautofluorescence of Photooxidized Bisretinoid

Another possible factor contributing to hyperautofluorescence at the fundus is lipofuscin photooxidation. The bisretinoids of lipofuscin (e.g., the all-*trans*-retinal dimer A2E) are photoreactive compounds that, on photon absorbance, can generate reactive forms of oxygen and become oxidized.^{13,19,63,64} Photooxidation is a process that is ongoing in the human eye since these photooxidized forms of A2E and all-*trans*-retinal dimer are present in isolated human RPE.^{13,64} As shown in Figure 3, in which A2E is used as an example, oxidation at one carbon-carbon double bond along the short-arm of the molecule results in a 6- to 13-fold increase in fluorescence intensity (calculated as fluorescence peak height/absorbance peak height; Fig. 3, peaks 1 and 3). Not only could photooxidized bisretinoid be a source of heightened fundus autofluorescence, it is a feature that should be considered when quantifying fundus autofluorescence intensity and extrapolating to RPE lipofuscin abundance. It is worth noting that oxidation of these bisretinoids at additional carbon-carbon double bonds, specifically those on the long arm of the molecule (Fig. 3, peak 2), could decrease the contribution to fundus autofluorescence. Again, in the case of A2E, this adjustment would occur because the hypsochromic shift (blue shift) associated with loss of a carbon-carbon double bond on the long arm of A2E, displaces the excitation maxima away from the 488-nm wavelength used by fundus autofluorescence imaging.

Heightened Autofluorescence Emanating from Photoreceptor Outer Segments

Photoreceptor cells are the sites of step-wise nonenzymatic synthesis of RPE lipofuscin fluorophores. The lipofuscin-associated pigments present in photoreceptor cell outer segments include A2-DHP-PE, all-*trans*-retinal dimer, all-*trans*-retinal dimer-PE, and A2PE. As discussed earlier, under healthy conditions, the bisretinoids are not amassed with abundance in photoreceptor cells. Instead the lipofuscin-burdened outer segment material is shed in packets for transfer to the RPE. Our ongoing studies of the RCS (Royal College of Surgeons) rat have revealed, however, that, at least under some conditions, bisretinoid formation in photoreceptor cells can be markedly increased (Fig. 4). The RCS rat exhibits a recessively inherited disorder wherein the RPE cells fail to phagocytose shed outer segment membrane; as a consequence, photoreceptor cells begin to degenerate.^{65–67} In RCS rat eyes 30 days after birth, rhodopsin levels are not yet reduced,⁶⁵ and outer nuclear layer thickness at the posterior pole is either unchanged⁶⁸ or is reduced by approximately 30%.⁶⁹ At this age, we found by HPLC quantitation that the bisretinoids A2PE and all-*trans*-retinal dimer were three- and sevenfold higher, respectively, than in the nonmutant rat (Fig. 4B). These findings indicate that in the presence of RPE-photoreceptor cell failure, the activity of the lipofuscin biosynthetic pathway can be strikingly elevated. Since these compounds form from reactions of all-*trans*-retinal, overactive bisretinoid synthesis probably results from inefficient clearance of all-*trans*-retinal by the photoreceptor cell. At least in part, this inefficiency could be rooted in the large amount of energy in the form of ATP and NADPH that is needed to recover from photoexcitation: ATP is essential in the mediation of translocation of all-*trans*-retinal to the cytosolic locale of all-*trans*-retinol dehydrogenase, and NADPH is

FIGURE 2. The fluorescence intensity of RPE lipofuscin bisretinoids increased as the abundance of the pigments was augmented. A2E and all-*trans*-retinal dimer were injected into a UPLC (ultra-performance liquid chromatography) system, with reversed-phase column, at the indicated amounts in a 5- μ L volume, and the samples were monitored with a fluorescence detector. UV-visible absorbances were monitored but are not shown. *Insets:* structures, and absorbance maxima (λ_{max}) of A2E and all-*trans*-retinal dimer.

FIGURE 3. Fluorescence intensity of RPE lipofuscin bisretinoids was increased after photooxidation on the short arm of the molecule. Samples of A2E were irradiated at 430 nm to generate photooxidation products (oxo-A2E 1, 2, and 3) and then analyzed by reversed-phase UPLC (ultra-performance liquid chromatography) with online monitoring of absorbance (black trace) and fluorescence (red trace). Fluorescence efficiency per absorbed photon, calculated as fluorescence peak height/absorbance peak height, was 83.6 for oxo-A2E 1, 36.1 for oxo-A2E 3, and 6.7 for A2E. Note that oxo-A2E 2 exhibited little or no fluorescence.

requisite in the reduction of all-*trans*-retinal to all-*trans*-retinol. For the needed NADPH, the reduction process must also contend with the glutathione redox cycle that in outer segments is solely responsible for protecting unsaturated fatty acids from hydrogen peroxide-mediated damage.⁷⁰ These observations do not just point to impaired photoreceptor cells as being a potential source of fundus autofluorescence; they also offer an explanation for why the aberrant autofluorescence derived from failing photoreceptor cells can exceed intensity levels originating from RPE situated elsewhere in the fundus—RPE cells that would have been accumulating lipofuscin over a lifetime.

There is an additional factor that could potentiate outer segment autofluorescence. When excited at 488 nm, A2PE, a fluorophore contributing to autofluorescence from outer segments, exhibits greater fluorescence intensity than does A2E (a fluorophore inside the RPE; Fig. 4B).

There is evidence to support the concept that the presence of increased fundus autofluorescence in the region surround-

ing an area of atrophy could, at least under some conditions, be an expression of ongoing photoreceptor cell dysfunction and degeneration. For instance, OCT findings in the zone of enhanced autofluorescence surrounding GA include thinning of the reflective band assumed to be RPE/Bruch's membrane, obscuring of the band corresponding to the inner segment-outer segment junction, and separation of the outer retinal bands from RPE/Bruch's membrane.^{43,71} Photoreceptor function is also impaired in these hyperautofluorescent regions.⁵² Increased autofluorescence associated with photoreceptor cell failure after RPE atrophy or loss, would also explain why areas of AF images exhibiting decreased or absent autofluorescence (RPE atrophy) can transition to areas of increased autofluorescence (photoreceptor cell failure with augmented bisretinoid formation), as has been described in *ABCA4*-associated Star-gard disease.⁷²

The concept that hyperautofluorescence in the perifoveal rings of RP originates from impaired photoreceptor outer segments is consistent with evidence demonstrating that photo-

FIGURE 4. Bisretinoid formation in impaired photoreceptors can greatly exceed that generated in healthy photoreceptor cell outer segments. (A) HPLC quantitation of all-*trans*-retinal (retinoid precursor of RPE lipofuscin) and two bisretinoids (all-*trans*-retinal dimer and A2PE) that form in photoreceptor cells via the lipofuscin biosynthetic pathway. Eyecups of RCS and control (RCS *rdy*+) albino rats, age 1 month, included RPE and neural retina. Under normal conditions, phospholipase D-mediated phosphate hydrolysis of A2PE (dashed line in structure) in RPE cell lysosomes releases A2E, and the latter then accumulates in RPE. However, in the RCS rat, because of the failure to phagocytose, most of the pigment generated within the A2PE/A2E pathway remains as A2PE. (B) Fluorescence emission spectra of A2E and A2PE recorded at an excitation of 488 nm. The slightly greater fluorescence intensity of A2PE probably reflects an excitation maximum (~449 nm) that is closer to 488 nm than the excitation maximum of A2E (~439 nm).

receptor light sensitivity measured over the abnormal annulus is reduced.^{5,54,55,73} Augmented bisretinoid in photoreceptor cells as a source of AF could also be an explanation for the observation that, in a form of autosomal recessive cone-rod dystrophy, AF imaged with conventional 488 nm excitation has been found to be considerably increased relative to normal, despite evidence of RPE atrophy as demonstrated by OCT and reduced near-infrared autofluorescence (787 nm excitation).⁶² The latter signal originates in large part from RPE melanin.⁷⁴

SUMMARY

We have demonstrated that augmented fundus autofluorescence in connection with retinal disease can arise, not just from an increase in lipofuscin in RPE, but from other mechanisms that include photooxidative modification of the RPE lipofuscin fluorophores; abnormally amplified bisretinoid synthetic activity in disabled photoreceptor cells; and a shift toward forms of the bisretinoids that exhibit excitation maxima closer to the 488-nm excitation typically used by fundus autofluorescence imaging. In AMD, RPE failure probably precedes photoreceptor cell degeneration,⁷⁵ yet RPE cell death would not necessarily have to be completed before photoreceptor cells also begin to die. In the period of RPE malfunctioning that portends RPE cell death, failure to phagocytose shed outer segment membrane is likely to result in a decline in photoreceptor cell functioning followed by degeneration, as seen in the RCS rat. Under these conditions, anomalous handling of retinoid, specifically reactive all-*trans*-retinal, could rapidly amplify bisretinoid formation in the degenerating outer segments. This discussion has emphasized the high-intensity autofluorescence that can be found at margins of GA. However, even in early stages of AMD, various patterns of increased fundus autofluorescence are observed, some of which take the form of discrete foci. Any or all the mechanisms discussed herein contribute to and may aid in interpreting the autofluorescence changes at specific stages of disease. Rather than signaling healthy photoreceptors providing lipofuscin to RPE, the parafoveal rings of RP likely reflect early stage photoreceptor cell degeneration marked by intensified bisretinoid formation due to vitamin A aldehyde mishandling by dysfunctioning photoreceptor cells.

Areas of hyperautofluorescence in the fundus are not necessarily indicative of the presence of RPE; nevertheless, the mechanisms described herein deserve further investigation. Understanding the nature of hyperautofluorescence in the fundus is important in determining its prognostic value. Moreover hyperautofluorescent regions may provide useful measures of therapeutic effectiveness as treatments become available.

Acknowledgments

The authors thank Claudia Keilhauer (Department of Ophthalmology, University Hospital, Würzburg, Germany) for contributions to Figure 1.

References

- Delori FC, Dorey CK, Staurenghi G, Arend O, Goger DG, Weiter JJ. In vivo fluorescence of the ocular fundus exhibits retinal pigment epithelium lipofuscin characteristics. *Invest Ophthalmol Vis Sci.* 1995;36:718–729.
- von Ruckmann A, Fitzke FW, Bird AC. Distribution of fundus autofluorescence with a scanning laser ophthalmoscope. *Br J Ophthalmol.* 1995;79:407–412.
- von Ruckmann A, Fitzke FW, Bird AC. In vivo fundus autofluorescence in macular dystrophies. *Arch Ophthalmol.* 1997;115:609–615.
- Holz FG, Bellman C, Staudt S, Schutt F, Volcker HE. Fundus autofluorescence and development of geographic atrophy in age-related macular degeneration. *Invest Ophthalmol Vis Sci.* 2001;42:1051–1056.
- Robson AG, Michaelides M, Saihan Z, et al. Functional characteristics of patients with retinal dystrophy that manifest abnormal parafoveal annuli of high density fundus autofluorescence: a review and update. *Doc Ophthalmol.* 2008;116:79–89.
- Delori FC. Spectrophotometer for noninvasive measurement of intrinsic fluorescence and reflectance of the ocular fundus. *Appl Opt.* 1994;33:7439–7452.
- Spaide RF. Autofluorescence imaging with the fundus camera. In: Holz FG, Schmitz-Valckenberg S, Spaide RF, Bird AC, eds. *Atlas of Fundus Autofluorescence Imaging*. Berlin-Heidelberg: Springer-Verlag; 2007:49–54.
- Morgan JJ, Hunter JJ, Masella B, et al. Light-induced retinal changes observed with high-resolution autofluorescence imaging of the retinal pigment epithelium. *Invest Ophthalmol Vis Sci.* 2008;49:3715–3729.
- von Ruckmann A, Fitzke FW, Bird AC. Fundus autofluorescence in age-related macular disease imaged with a laser scanning ophthalmoscope. *Invest Ophthalmol Vis Sci.* 1997;38:478–486.
- Delori FC, Goger DG, Dorey CK. Age-related accumulation and spatial distribution of lipofuscin in RPE of normal subjects. *Invest Ophthalmol Vis Sci.* 2001;42:1855–1866.
- Delori FC, Keilhauer C, Sparrow JR, Staurenghi G. Origin of fundus autofluorescence. In: Holz FG, Schmitz-Valckenberg S, Spaide RF, Bird AC, eds. *Atlas of Fundus Autofluorescence Imaging*. Berlin-Heidelberg: Springer-Verlag; 2007:17–29.
- Del Priore LV, Kuo YH, Tezel TH. Age-related changes in human RPE cell density and apoptosis proportion in situ. *Invest Ophthalmol Vis Sci.* 2002;43:3312–3318.
- Kim SR, Jang Y, Sparrow JR. Photooxidation of RPE lipofuscin bisretinoids enhanced fluorescence intensity. *Vision Res.* 2010;50:729–736.
- Sparrow JR. Lipofuscin of the retinal pigment epithelium. In: Holz FG, Schmitz-Valckenberg S, Spaide RF, Bird AC, eds. *Atlas of Fundus Autofluorescence Imaging*. Berlin-Heidelberg: Springer-Verlag; 2007:3–16.
- Ng KP, Gugiu BG, Renganathan K, et al. Retinal pigment epithelium lipofuscin proteomics. *Mol Cell Proteomics.* 2008;7:1397–1405.
- Lorenz B, Wabbel B, Wegscheider E, Hamel CP, Drexler W, Presing MN. Lack of fundus autofluorescence to 488 nanometers from childhood on in patients with early-onset severe retinal dystrophy associated with mutations in RPE65. *Ophthalmology.* 2004;111:1585–1594.
- Parish CA, Hashimoto M, Nakanishi K, Dillon J, Sparrow JR. Isolation and one-step preparation of A2E and iso-A2E, fluorophores from human retinal pigment epithelium. *Proc Natl Acad Sci U S A.* 1998;95:14609–14613.
- Fishkin N, Sparrow JR, Allikmets R, Nakanishi K. Isolation and characterization of a retinal pigment epithelial cell fluorophore: an all-*trans*-retinal dimer conjugate. *Proc Natl Acad Sci U S A.* 2005;102:7091–7096.
- Kim SR, Jang YP, Jockusch S, Fishkin NE, Turro NJ, Sparrow JR. The all-*trans*-retinal dimer series of lipofuscin pigments in retinal pigment epithelial cells in a recessive Stargardt disease model. *Proc Natl Acad Sci U S A.* 2007;104:19273–19278.
- Wu Y, Fishkin NE, Pande A, Pande J, Sparrow JR. Novel lipofuscin bisretinoids prominent in human retina and in a model of recessive Stargardt disease. *J Biol Chem.* 2009;284:20155–20166.
- Liu J, Itagaki Y, Ben-Shabat S, Nakanishi K, Sparrow JR. The biosynthesis of A2E, a fluorophore of aging retina, involves the formation of the precursor, A2-PE, in the photoreceptor outer segment membrane. *J Biol Chem.* 2000;275:29354–29360.
- Ben-Shabat S, Parish CA, Vollmer HR, et al. Biosynthetic studies of A2E, a major fluorophore of RPE lipofuscin. *J Biol Chem.* 2002;277:7183–7190.
- Kim SR, He J, Yanase E, et al. Characterization of dihydro-A2PE: an intermediate in the A2E biosynthetic pathway. *Biochemistry.* 2007;46:10122–10129.
- Bok D. Retinal photoreceptor-pigment epithelium interactions: The Friedenwald Lecture. *Invest Ophthalmol Vis Sci.* 1985;26:1659–1694.

25. Mata NL, Weng J, Travis GH. Biosynthesis of a major lipofuscin fluorophore in mice and humans with ABCR-mediated retinal and macular degeneration. *Proc Natl Acad Sci U S A*. 2000;97:7154-7159.
26. Radu RA, Mata NL, Nusinowitz S, Liu X, Sieving PA, Travis GH. Treatment with isotretinoin inhibits lipofuscin and A2E accumulation in a mouse model of recessive Stargardt's macular degeneration. *Proc Natl Acad Sci U S A*. 2003;100:4742-4747.
27. Kim SR, Fishkin N, Kong J, Nakanishi K, Allikmets R, Sparrow JR. The Rpe65 Leu450Met variant is associated with reduced levels of the RPE lipofuscin fluorophores A2E and iso-A2E. *Proc Natl Acad Sci U S A*. 2004;101:11668-11672.
28. Radu RA, Han Y, Bui TV, et al. Reductions in serum vitamin A arrest accumulation of toxic retinal fluorophores: a potential therapy for treatment of lipofuscin-based retinal diseases. *Invest Ophthalmol Vis Sci*. 2005;46:4393-4401.
29. Maiti P, Kong J, Kim SR, Sparrow JR, Allikmets R, Rando RR. Small molecule RPE65 antagonists limit the visual cycle and prevent lipofuscin formation. *Biochemistry*. 2006;45:852-860.
30. Maeda A, Maeda T, Sun W, Zhang H, Baehr W, Palczewski K. Redundant and unique roles of retinol dehydrogenases in the mouse retina. *Proc Natl Acad Sci U S A*. 2007;104:19565-19570.
31. Molday LL, Rabin AR, Molday RS. ABCR expression in foveal cone photoreceptors and its role in Stargardt macular dystrophy. *Nat Genet*. 2000;25:257-258.
32. Papermaster DS, Schneider BG, Zorn MA, Kraehenbuhl JP. Immunocytochemical localization of a large intrinsic membrane protein to the incisures and margins of frog rod outer segment disks. *J Cell Biol*. 1978;78:415-425.
33. Sun H, Nathans J. Stargardt's ABCR is localized to the disc membrane of retinal rod outer segments. *Nat Genet*. 1997;17:15-16.
34. Sun H, Nathans J. ABCR, the ATP-binding cassette transporter responsible for Stargardt macular dystrophy, is an efficient target of all-trans retinal-mediated photo-oxidative damage in vitro: implications for retinal disease. *J Biol Chem*. 2001;276:11766-11774.
35. Allikmets R, Singh N, Sun H, et al. A photoreceptor cell-specific ATP-binding transporter gene (ABCR) is mutated in recessive Stargardt macular dystrophy. *Nat Genet*. 1997;15:236-246.
36. Shroyer NF, Lewis RA, Allikmets R, et al. The rod photoreceptor ATP-binding cassette transporter gene, ABCR, and retinal disease: from monogenic to multifactorial. *Vision Res*. 1999;39:2537-2544.
37. Eagle RC, Lucier AC, Bernardino VB, Yanoff M. Retinal pigment epithelial abnormalities in fundus flavimaculatus. *Ophthalmology*. 1980;87:1189-1200.
38. Weng J, Mata NL, Azarian SM, Tzekov RT, Birch DG, Travis GH. Insights into the function of Rim protein in photoreceptors and etiology of Stargardt's disease from the phenotype in abcr knockout mice. *Cell*. 1999;98:13-23.
39. Delori FC, Staurenghi G, Arend O, Dorey CK, Goger DG, Weiter JJ. In vivo measurement of lipofuscin in Stargardt's disease-Fundus flavimaculatus. *Invest Ophthalmol Vis Sci*. 1995;36:2327-2331.
40. Lois N, Holder GE, Bunce CV, Fitzke FW, Bird AC. Phenotypic subtypes of Stargardt macular dystrophy-fundus flavimaculatus. *Arch Ophthalmol*. 2001;119:359-369.
41. Chrispell JD, Feathers KL, Kane MA, et al. Rdh12 activity and effects on retinoid processing in the murine retina. *J Biol Chem*. 2009;284:21468-21477.
42. Schmitz-Valckenberg S, Jorzik J, Unnebrink K, Holz FG. Analysis of digital scanning laser ophthalmoscopy fundus autofluorescence images of geographic atrophy in advanced age-related macular degeneration. *Graefes Arch Clin Exp Ophthalmol*. 2002;240:73-78.
43. Fleckenstein M, Issa PC, Helb HM, et al. High-resolution spectral domain-OCT imaging in geographic atrophy associated with age-related macular degeneration. *Invest Ophthalmol Vis Sci*. 2008;49:4137-4144.
44. Sunness JS, Gonzalez-Baron J, Applegate CA, et al. Enlargement of atrophy and visual acuity loss in the geographic atrophy form of age-related macular degeneration. *Ophthalmology*. 1999;106:1768-1779.
45. Holz FG, Bellmann C, Margaritis M, Schutt F, Otto TP, Volcker HE. Patterns of increased in vivo fundus autofluorescence in the junctional zone of geographic atrophy of the retinal pigment epithelium associated with age-related macular degeneration. *Graefes Arch Clin Exp Ophthalmol*. 1999;237:145-152.
46. Dreyhaupt J, Mansmann U, Pritsch M, Dolar-Szczasny J, Bindewald A, Holz FG. Modelling the natural history of geographic atrophy in patients with age-related macular degeneration. *Ophthalmic Epidemiol*. 2005;12:353-362.
47. Holz FG, Bindewald-Wittich A, Fleckenstein M, et al. Progression of geographic atrophy and impact of fundus autofluorescence patterns in age-related macular degeneration. *Am J Ophthalmol*. 2007;143:463-472.
48. Hwang JC, Chan JW, Chang S, Smith RT. Predictive value of fundus autofluorescence for development of geographic atrophy in age-related macular degeneration. *Invest Ophthalmol Vis Sci*. 2006;47:2655-2661.
49. Bindewald A, Bird AC, Dandekar SS, et al. Classification of fundus autofluorescence patterns in early age-related macular disease. *Invest Ophthalmol Vis Sci*. 2005;46:3309-3314.
50. Bindewald A, Schmitz-Valckenberg S, Jorzik JJ, et al. Classification of abnormal fundus autofluorescence patterns in the junctional zone of geographic atrophy in patients with age related macular degeneration. *Br J Ophthalmol*. 2005;89:874-878.
51. Scholl HPN, Bellmann C, Dandekar SS, Bird AC, Fitzke FW. Photopic and scotopic fine matrix mapping of retinal areas of increased fundus autofluorescence in patients with age-related maculopathy. *Invest Ophthalmol Vis Sci*. 2004;45:574-583.
52. Schmitz-Valckenberg S, Bultmann S, Dreyhaupt J, Bindewald A, Holz FG, Rohrschneider K. Fundus autofluorescence and fundus perimetry in the junctional zone of geographic atrophy in patients with age-related macular degeneration. *Invest Ophthalmol Vis Sci*. 2004;45:4470-4476.
53. Robson AG, Michaelides M, Luong VA, et al. Functional correlates of fundus autofluorescence abnormalities in patients with RPGR or RMS1 mutations causing cone or cone rod dystrophy. *Br J Ophthalmol*. 2008;92:95-102.
54. Robson AG, Saihan Z, Jenkins SA, et al. Functional characterisation and serial imaging of abnormal fundus autofluorescence in patients with retinitis pigmentosa and normal visual acuity. *Br J Ophthalmol*. 2006;90:472-479.
55. Popovic P, Jarc-Vidmar M, Hawlina M. Abnormal fundus autofluorescence in relation to retinal function in patients with retinitis pigmentosa. *Graefes Arch Clin Exp Ophthalmol*. 2005;243:1018-1027.
56. Michaelides M. Fundus autofluorescence in cone and cone-rod dystrophies. In: Lois N, Forrester JV, eds. *Fundus Autofluorescence*. Philadelphia: Lippincott Williams and Wilkins; 2009:153-166.
57. Gomes NL, Greenstein VC, Carlson JN, et al. A comparison of fundus autofluorescence and retinal structure in patients with Stargardt disease. *Invest Ophthalmol Vis Sci*. 2009;50:3953-3959.
58. Lois N, Halfyard A, Bird AC, Fitzke FW. Quantitative evaluation of fundus autofluorescence imaged 'in vivo' in eyes with retinal disease. *Br J Ophthalmol*. 2000;84:741-745.
59. Cideciyan AV, Aleman TS, Swider M, et al. Mutations in ABCA4 result in accumulation of lipofuscin before slowing of the retinoid cycle: a reappraisal of the human disease sequence. *Hum Mol Genet*. 2004;13:525-534.
60. Sparrow JR, Boulton M. RPE lipofuscin and its role in retinal photobiology. *Exp Eye Res*. 2005;80:595-606.
61. Holz FG, Fleckenstein M, Schmitz-Valckenberg S, Bird AC. Evaluation of fundus autofluorescence images. In: Holz FG, Schmitz-Valckenberg S, Spaide RF, Bird AC, eds. *Atlas of Fundus Autofluorescence Imaging*. Berlin-Heidelberg: Springer-Verlag; 2007:71-76.
62. Aleman TS, Soumitra N, Cideciyan AV, et al. CERKL mutations cause an autosomal recessive cone-rod dystrophy with inner retinopathy. *Invest Ophthalmol Vis Sci*. 2009 50:5944-5954.
63. Ben-Shabat S, Itagaki Y, Jockusch S, Sparrow JR, Turro NJ, Nakanishi K. Formation of a nona-oxirane from A2E, a lipofuscin fluorophore related to macular degeneration, and evidence of singlet oxygen involvement. *Angew Chem Int Ed Engl*. 2002;41:814-817.
64. Jang YP, Matsuda H, Itagaki Y, Nakanishi K, Sparrow JR. Characterization of peroxy-A2E and furan-A2E photooxidation products

- and detection in human and mouse retinal pigment epithelial cells lipofuscin. *J Biol Chem.* 2005;280:39732–39739.
65. Dowling JE, Sidman RL. Inherited retinal dystrophy in the rat. *J Cell Biol.* 1962;14:73–109.
 66. Matthes MT, La Vail MM. Inherited retinal dystrophy in the RCS rat: composition of the outer segment debris zone. *Prog Clin Biol Res.* 1989;314:315–330.
 67. D'Cruz PM, Yasumura D, Weir J, et al. Mutation of the receptor tyrosine kinase gene *Mertk* in the retinal dystrophic RCS rat. *Hum Mol Genet.* 2000;9:645–651.
 68. Jolly C, Jeanny JC, Behar-Cohen F, Laugier P, Saied A. High-resolution ultrasonography of subretinal structure and assessment of retina degeneration in rat. *Exp Eye Res.* 2005;81:592–601.
 69. LaVail MM, Battelle B-A. Influence of eye pigmentation and light deprivation on inherited retinal dystrophy in the rat. *Exp Eye Res.* 1975;21:167.
 70. Hsu S, Molday RS. Glucose metabolism in photoreceptor outer segments. *J Biol Chem.* 1994;269:17954–17959.
 71. Wolf-Schnurrbusch UEK, Enzmann V, Brinkmann CK, Wolf S. Morphological changes in patients with geographic atrophy assessed with a novel spectral OCT-SLO combination. *Invest Ophthalmol Vis Sci.* 2008;49:3095–3099.
 72. Smith RT, Gomes NL, Barile G, Busuioc M, Lee N, Laine A. Lipofuscin and autofluorescence metrics in progressive STGD. *Invest Ophthalmol Vis Sci.* 2009;50:3907–3914.
 73. Robson AG, Egan CA, Luong VA, Bird AC, Holder GE, Fitzke FW. Comparison of fundus autofluorescence in photopic and scotopic fine-matrix mapping in patients with retinitis pigmentosa and normal visual acuity. *Invest Ophthalmol Vis Sci.* 2004;45:4119–4125.
 74. Keilhauer CN, Delori FC. Near-infrared autofluorescence imaging of the fundus: visualization of ocular melanin. *Invest Ophthalmol Vis Sci.* 2006;47:3556–3564.
 75. Sarks JP, Sarks SH, Killingsworth MC. Evolution of geographic atrophy of the retinal pigment epithelium. *Eye.* 1988;2:552–577.