Comparison of Near-Infrared and Short-Wavelength Autofluorescence in Retinitis Pigmentosa

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PURPOSE. To compare near-infrared autofluorescence (NIR-AF) and short-wavelength (SW) AF in retinitis pigmentosa (RP) and assess their relationships to underlying retinal structure and visual function.

METHODS. SW-AF, NIR-AF, and spectral domain optical coherence tomography (SD-OCT) images were acquired from 31 patients (31 eyes) with RP and registered to each other. Microperimetry was performed on a subset of 12 patients. For both SW-AF and NIR-AF images, three independent observers measured the area enclosed by the outer border of the hyperautofluorescent ring and the distance from the fovea to the outer and inner border of the ring. For SD-OCT images, the distance from the fovea to the location where the inner segment ellipsoid (ISe) band became undetectable was measured.

RESULTS. All eyes had a hyperautofluorescent ring on both SW-AF and NIR-AF. The position of the outer border of the ring was similar for both modalities. On NIR-AF the signal outside the ring was lower than inside the ring, resulting in a high contrast between the two areas. Also, the inner border of the ring was closer to the fovea on NIR-AF than SW-AF, corresponding to a location on SD-OCT where the ISe band was at least partially intact. Visual sensitivity was relatively preserved within the ring, reduced across the ring, and markedly decreased or nonrecordable outside the ring.

CONCLUSIONS. SW-AF and NIR-AF are both useful for monitoring disease progression in RP; however, NIR-AF may have advantages clinically and could unveil a process that precedes the formation of fluorophores that emit the SW-AF signal. (*Invest Ophthalmol Vis Sci.* 2013;54:585–591) DOI:10.1167/iov.12-11176

Retinitis pigmentosa (RP) encompasses a genetically heterogeneous group of retinal degenerations characterized by progressive loss of photoreceptors and visual function. The

disease typically starts in the peripheral retina and progresses toward the fovea, leading to severe visual loss in advanced stages.

A number of noninvasive imaging techniques have been proposed for monitoring progression of RP and for aiding in the distinction between “healthy, functional” and “unhealthy, dysfunctional” retinal areas. Two examples of imaging modalities are short-wavelength autofluorescence (SW-AF) and near-infrared autofluorescence (NIR-AF) imaging. The signal for SW-AF (488 nm excitation) is derived primarily from lipofuscin of the retinal pigment epithelium (RPE),¹ with the lipofuscin fluorophores responsible for SW-AF originating in photoreceptor cells from inadvertent reactions of all-*trans*-retinal.² In patients with RP, SW-AF often reveals a ring or arc of high AF that encloses an AF signal interior to the ring that appears qualitatively normal. The inner border of the high AF ring has been found to correspond spatially with the lateral extent of a preserved hyperreflective band attributable to the inner segment ellipsoid (ISe) on spectral domain optical coherence tomography (SD-OCT).³ Although recent studies have shown that cone photoreceptor loss can also occur within the ring,⁴ cone-mediated visual function is relatively preserved in this area, hence AF is reported to be a useful tool for monitoring disease progression.⁵

Although SW-AF is being increasingly used as a diagnostic and prognostic tool in RP, the clinical use of NIR-AF is less widespread. NIR-AF (787 nm excitation) originates from melanin in RPE and choroid.⁶ Contributions of other fluorophores are still under debate.⁷ Previous studies have shown that NIR-AF can also reveal a ring or arc in RP similar to that demonstrated with SW-AF.⁸ In this study, we compare NIR-AF and SW-AF in a cohort of RP patients and assess the relationships of these signals to underlying retinal structure and visual function.

METHODS

Subjects

Thirty-one patients with a clinical diagnosis of RP were included in this cross-sectional study. Ages ranged from 7 to 68 years, with a mean age of 31 years (± 16 years). The clinical diagnosis of RP was based on typical fundus features, symptoms, and family history. The diagnosis was confirmed with full-field electroretinography testing following International Society for Clinical Electrophysiology of Vision standards.⁹ Eyes were excluded from the study if there was evidence of cystic macular edema, epiretinal membranes, significant cataract, or a history of other ocular diseases (e.g., glaucoma, diabetes). One eye from each patient was chosen for analysis based on image quality and exclusion criteria. With the exception of two patients, P7 and P24, refractive errors of tested eyes did not exceed ± 5 diopters spherical. All procedures adhered to the tenets of the Declaration of Helsinki, and written informed consent was obtained from all subjects after a full

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TABLE 1. Summary of Demographic and Clinical Findings

Patient	Inheritance Pattern	Sex	Age, y	Eye	Iris Color	BCVA Snellen (logMAR)
1	Usher II	F	14	OS	Blue	20/20 (0.0)
2	AR	F	28	OS	Brown	20/20 (0.0)
3	AR	F	26	OS	Blue	20/20 (0.0)
4	Usher II	M	20	OD	Blue	20/40 (0.3)
5	AD	M	53	OD	Green	20/25 (0.1)
6	Usher II	F	25	OS	Brown	20/25 (0.1)
7	AR	F	52	OD	Brown	20/30 (0.2)
8	AD	M	36	OS	Blue	20/20 (0.0)
9*	Usher I	M	7	OS	Brown	20/30 (0.2)
10*	Usher I	F	10	OD	Brown	20/25 (0.1)
11	AD	M	58	OS	Brown	20/25 (0.1)
12	AD	M	50	OD	Hazel	20/20 (0.0)
13	AR	M	22	OS	Green	20/25 (0.1)
14	AR	M	33	OD	Brown	20/30 (0.2)
15	Usher II	F	32	OD	Blue	20/20 (0.0)
16	AR	F	16	OS	Brown	20/20 (0.0)
17	AD	M	19	OD	Brown	20/25 (0.1)
18	AD	F	30	OD	Brown	20/20 (0.0)
19	AR	M	32	OD	Brown	20/20 (0.0)
20	AR	F	31	OD	Brown	20/20 (0.0)
21	AD	M	56	OD	Blue	20/20 (0.0)
22	Usher II	M	20	OD	Blue	20/20 (0.0)
23	AR	M	68	OD	Hazel	20/20 (0.0)
24	AR	M	37	OD	Brown	20/20 (0.0)
25	AD	M	28	OS	Blue	20/20 (0.0)
26	AR	F	17	OS	Brown	20/25 (0.1)
27	Usher II	F	37	OS	Hazel	20/25 (0.1)
28	AR	M	60	OS	Brown	20/20 (0.0)
29	X-Linked	M	30	OS	Brown	20/30 (0.2)
30	X-Linked	M	13	OD	Blue	20/30 (0.2)
31	AD	F	13	OD	Blue	20/30 (0.2)

AR, autosomal recessive; AD, autosomal dominant; BCVA, best corrected visual acuity; logMAR, logarithm of the minimum angle of resolution.

* Same family.

explanation of the procedures was provided. The protocol was approved by the institutional review board of Columbia University.

The mode of inheritance within the patient group included the following: autosomal dominant ($n = 9$), autosomal recessive ($n = 12$), Usher syndrome type I ($n = 2$), Usher syndrome type II ($n = 6$), and X-linked ($n = 2$) (see Table 1 for a summary of demographic and clinical findings).

Imaging

Pupils were dilated with topical 1% tropicamide and 2.5% phenylephrine. SW-AF images (30°) of the fundus and corresponding horizontal 9-mm SD-OCT scans through the fovea were recorded simultaneously with the Spectralis scanning laser ophthalmoscope (Spectralis HRA-OCT; Heidelberg Engineering, Heidelberg, Germany) after 20 seconds of bleaching of photopigments.¹⁰ NIR-AF images (30°) were acquired with the Heidelberg Retina Angiograph 2 scanning laser ophthalmoscope (HRA2-SLO; Heidelberg Engineering) using the indocyanine-green angiography mode (without dye injection) after adjusting the focus in near-infrared reflectance mode. The eye-tracking feature of both SLOs was used to obtain images with improved signal-to-noise ratio through averaging of scans. NIR-AF and SW-AF images were recorded using the normalized mode (automatic histogram stretching for contrast enhancement). Both image modalities were registered to each other in Photoshop CS5 (Adobe, San Jose, CA).

For a subgroup of 12 patients, microperimetry was performed before AF imaging with the Nidek MP-1 (NAVIS software version 1.7.3; Nidek Technologies, Padua, Italy). The patients, who had recent experience of at least one visual field test, were adapted to the

test background for 20 minutes following pupil dilation. The MP-1 pattern consisted of 68 test locations within 10° of the fovea with a separation of 2° between the stimulus locations. The 10-2 pattern was similar to the Humphrey 10-2 visual field pattern. The nontested eye was occluded. White test lights (stimulus size Goldmann III, 200 ms in duration) were presented on a 1.27-cd/m² white background using a 4-2 threshold strategy. Subjects were asked to maintain fixation on a 2° red cross. The microperimetry results were manually registered to the imported 30° SW-AF and NIR-AF images in the Nidek MP-1 using NAVIS software (Nidek Technologies), and the localized sensitivities for each patient were compared with the SW-AF and NIR-AF images.

Analyses

All the SW-AF and NIR-AF images and the corresponding SD-OCT scans were analyzed by three independent observers (TD, MRT, WL). The ruler tool in Photoshop CS5 (Adobe) was used to obtain measurements in millimeters. It was assumed that the image width (1536 pixels for SD-OCT scans and 768 pixels for AF images) was equivalent to 9 mm. On SD-OCT scans, the distance from the center of the fovea along the horizontal meridian to the location where the hyper-reflective ISe band, formerly referred to as the inner segment/outer segment (IS/OS) junction,¹¹ was no longer visible was measured. An example is shown in Figures 1C, 1D together with SW-AF and NIR-AF images; the white vertical dashed lines on the SD-OCT image indicate the position where the ISe band is no longer visible. On the registered AF images, the distances from the foveal center to the inner and to the outer border of the high AF ring were measured along the horizontal

FIGURE 1. SW-AF and NIR-AF images (A, B) and the corresponding horizontal SD-OCT line scan (C, D) for P16. The *horizontal white lines with single-headed arrows* in the SW-AF and NIR-AF images indicate the position of the corresponding horizontal SD-OCT scan. In the images of the SD-OCT scan (C, D), the *vertical white dashed lines* indicate the position where the ISe band is no longer visible. The latter is indicated in the SW-AF (A) and NIR-AF (B) images by the *black and white vertical dashed lines*, respectively. In the image of the SD-OCT scan (D), the distance from the fovea to the location where the ISe band is no longer visible is indicated by the *white double-headed arrow*. In the SW-AF and NIR-AF images (A, B), the distance from the fovea to the inner and to the outer border of the ring is indicated by the *white dashed double-headed arrows*.

axis (see dashed arrows in Figs. 1A, 1B). The outline of the AF ring was traced in ImageJ software (provided in the public domain by National Institutes of Health, Bethesda, MD; available at <http://imagej.nih.gov/ij/>) to measure the total ring-area in mm². Four patients were not included in the area measurements because their rings extended beyond the 30° field. Bland-Altman plots were generated (GraphPad Prism 5; GraphPad Software, La Jolla, CA) to assess the agreement between SW-AF and NIR-AF measurements. Statistical significance of the differences was tested with a paired samples test in SPSS Statistics 20.0 for Mac (SPSS IBM, Chicago, IL). Interclass correlation coefficients (ICC) were calculated in SPSS to assess statistical agreement between observers.

RESULTS

In each of the 31 eyes included in the study, both SW-AF and NIR-AF imaging revealed an autofluorescent ring (or arc) in the 30° field. Examples of SW-AF (upper row) and NIR-AF (lower row) images obtained from seven patients and a healthy control are shown in Figures 2 and 3. They have been chosen to illustrate the range of shapes and extent of rings/arcs in this group of patients. In the normal retina (Fig. 2A) the SW-AF signal is attenuated centrally by the absorption of the excitation light by macular pigment and melanin. Outside the macula, the AF signal is relatively homogeneous and reflects the distribution of RPE lipofuscin. The optic disc and blood

FIGURE 2. SW-AF and NIR-AF images obtained from a healthy control subject and from patients P15, P18, and P26. The SW-AF images are shown in the upper row (A–D) and the NIR-AF images are in the lower row (E–H). (A, E) The SW-AF and NIR-AF images for the normal retina.

FIGURE 3. SW-AF and NIR-AF images showing variably sized rings for patients P5, P14, P17, and P25. The SW-AF images are shown in the *upper row* (A–D) and the NIR-AF images are in the *lower row* (E–H).

vessels are dark due to absent AF emission and blockage of the AF signal, respectively. The corresponding NIR-AF image (Fig. 2E) is almost an inversion of SW-AF. Centrally, the signal from RPE melanin is greater, resulting in an increased NIR-AF signal. The extent and shape of the rings in the cohort of patients were similar for both SW-AF and NIR-AF, as can be seen in the examples presented in Figures 2 and 3. It was usually possible to identify an inner and outer border of the ring. On SW-AF, for most of the patients ($n = 26$) the intensity of the AF signal appeared to be higher across the ring than in the area outside the outer border. The intensity of the AF signal appeared to be at its lowest in the interior of the ring. On NIR-AF, the signal outside the ring was lower than that inside the ring, resulting in a high contrast between the two areas. In addition, in NIR-AF images, the signal across the ring was generally slightly higher

than the area interior to the ring. On NIR-AF the choroidal vasculature was often visible outside the ring.

To assess the relationship between visual sensitivity and AF, the 10-2 MP-1 results were superimposed on the 30° SW-AF and NIR-AF images. Figure 4 shows MP-1 results, in dB, superimposed on the 30° AF images for four patients (P2, P12, P21, P27). A higher dB value corresponds to better sensitivity. The dynamic range of the MP-1 is 2 log units, therefore the highest value is 20 dB and the lowest is 0 dB. Sensitivity values along the horizontal midline were calculated for each tested eye. As the 10-2 pattern has no test locations along the midline, 10 midline locations were assigned interpolated sensitivity values derived from actual values of the 10 test locations lying 1° above and 1° below each midline location. Sensitivities were relatively preserved interior to the ring (mean 14.1 ± 3.8 dB

FIGURE 4. MP-1 10-2 results in dB superimposed on the 30° SW-AF (*upper row*) and NIR-AF (*lower row*) images for 4 patients: P2, P12, P21, and P27. The *red cross* is the fixation cross for the MP-1 test and the *small turquoise points* overlying the cross represent the fixation pattern during testing.

TABLE 2. ICC for Measurements in SW-AF and NIR-AF Images

	SW-AF	NIR-AF
Ring area	0.999 (0.998–1.000)	0.999 (0.999–1.000)
Outer border temporal*	0.948 (0.905–0.973)	0.996 (0.992–0.998)
Outer border nasal*	0.977 (0.953–0.989)	0.989 (0.979–0.995)
Inner border temporal*	0.997 (0.994–0.998)	0.993 (0.987–0.996)
Inner border nasal*	0.981 (0.964–0.991)	0.993 (0.987–0.996)

The 95% confidence intervals are in parentheses.

* Measurements were obtained from fovea to corresponding border.

and 15.1 ± 4.1 dB for SW-AF and NIR-AF, respectively) but they were decreased across the ring (mean 9.2 ± 3.8 dB and 11.4 ± 4.1 dB for SW-AF and NIR-AF, respectively). Visual sensitivities outside the ring were markedly decreased or nonrecordable (mean 3.3 ± 4.6 dB, median 0.7 dB for SW-AF; mean 3.4 ± 4.6 dB, median 0.7 dB for NIR-AF).

A paired *t*-test analysis was used to compare the measurements that were obtained from SW-AF and NIR-AF images and the SD-OCT scans (see Table 2). There was no significant difference between the total ring-area measured on SW-AF and NIR-AF ($P = 0.197$). There was also no significant difference between the measurements for the outer temporal ring border ($P = 0.088$); however the distance from the fovea to the outer nasal border was significantly smaller on NIR-AF than SW-AF ($P = 0.043$), as were the distances from the fovea to the inner border of the ring for both the nasal and the temporal side ($P = 0.004$ and $P = 0.000$, respectively). The Bland-Altman plots in Figure 5 illustrate the differences in SW-AF and NIR-AF measurements from the fovea to the outer (Fig. 5A) and inner temporal (Fig. 5B) borders. For the outer temporal border measurements, the majority of data points are located around the zero axis, indicating a similarity between the SW-AF and NIR-AF measurements. For the inner temporal border of the hyperfluorescent ring, the majority of data points are located above the zero axis, indicating a decreased distance between the fovea and inner temporal border of the ring on NIR-AF.

We were also interested in comparing the location of ISe loss seen on SD-OCT with the location of the inner border of the ring measured on SW-AF and NIR-AF. There were no significant differences between measurements of the inner border of the ring on SW-AF and the location of ISe loss on SD-OCT for the temporal and nasal side ($P = 0.928$ and $P = 0.062$, respectively). However, for NIR-AF, the inner border of the ring was significantly closer to the fovea than the location of the ISe loss on SD-OCT for both the temporal and nasal side ($P = 0.000$ and $P = 0.000$, respectively).

ICCs revealed excellent agreement between observers for both SW-AF and NIR-AF (Table 2).

DISCUSSION

Both SW-AF and NIR-AF imaging revealed hyperautofluorescent rings in the RP patients. The location of the outer border of the ring in SW-AF images exhibited good correspondence with the location detected by NIR-AF. Consistent with previous reports comparing visual field results to changes in fundus AF,^{4,8,12} visual sensitivity was relatively preserved interior to the ring on SW-AF and NIR-AF, decreased on the ring, and markedly decreased or nonrecordable outside the outer border of the ring. However, we also found differences between the results of the two imaging modalities. For example, the inner border of the ring on NIR-AF was closer to the fovea than on SW-AF. This finding is intriguing, as the inner border coincided with a location where the hyperreflective ISe band on SD-OCT appeared to be at least partially preserved, if not intact.

FIGURE 5. Bland-Altman plots of the mean differences in measurements of the distance from the fovea to the outer (A) and inner temporal border (B) of the hyperautofluorescent ring on SW-AF and NIR-AF. (A) For the outer border of hyperautofluorescent ring the majority of data points are located around the zero axis, indicating no difference between SW-AF and NIR-AF measurements. (B) For the inner border of the hyperautofluorescent ring, the majority of data points are located above the zero axis, indicating that the distance between the fovea and inner border of the ring on NIR-AF is shorter. The dashed horizontal lines represent the 95% limits of agreement.

Therefore it is possible that NIR-AF could unveil a process in the progression of the disease that precedes the formation of fluorophores that lead to the SW-AF signal. In fact, a recent study of RP patients using SW-AF⁴ showed that the thickness values of the total receptor plus layer (Bruch's membrane to the border of the outer plexiform/inner nuclear layer) and the receptor outer segment plus layer (Bruch's membrane to the ISe band) were significantly decreased compared with values from healthy controls at distances that were closer to the fovea than the inner border of hyperautofluorescence. For patients with smaller-diameter rings, however, macular pigment absorbance of the SW-AF signal in this region cannot be ruled out as a contributing factor to the observed position of the inner border on SW-AF. Perhaps the most striking difference between the two modalities is the abrupt loss of the NIR-AF signal outside the ring, the latter demarcating healthier from less healthy retina.

What are the possible molecular correlates of the anomalous pattern of SW-AF and NIR-AF signals in this group of RP patients? It is well established that the signal for SW-AF is derived from retinal lipofuscin, a complex mixture of bisretinoids that originate in photoreceptor cells from reactions of all-*trans*-retinal.² These lipofuscin bisretinoid compounds are transferred to RPE secondarily. It has been suggested that the abnormal autofluorescence constituting the hyperautofluorescent ring indicates accelerated photoreceptor phagocytosis.^{12–14} However, the rate of formation of the

bisretinoid fluorophores is determined in photoreceptor cells before outer segment shedding and phagocytosis. We have previously suggested that disease-related patterns of hyperautofluorescence in SW-AF images may be indicative of a lipofuscin synthetic pathway that is accelerated because the disabled photoreceptor cells are unable to meet the demand for detoxification of all-*trans*-retinal, the latter being generated by photoisomerization of visual pigment.^{15,16} That photoreceptor cells within the borders of the hyperautofluorescent ring were impaired in the present study was indicated by decreased visual sensitivities on the MP-1, and loss of the ISE band on SD-OCT across the width of the AF ring. The source of the aberrant SW-AF across the ring could be the photoreceptor cells themselves, degenerating photoreceptor debris, or RPE cells after phagocytic transfer of the fluorophores. Bisretinoid lipofuscin compounds residing in photoreceptor cells may contribute comparatively brighter AF because the fluorescence would be unimpeded by melanin absorption of the exciting and emitted light, as is the case for RPE SW-AF.⁶ Recently developed approaches for quantifying the SW-AF autofluorescence signal¹⁰ will help to better define the areas of increased and decreased signal in future studies of RP patients.

The spatial correspondence between the hyperautofluorescent rings visible in the NIR-AF and SW-AF images is difficult to interpret given our current knowledge regarding the origin of NIR-AF (787 nm excitation). Studies of NIR-AF have led to the conclusion that the emission originates from melanin in the RPE and choroid with the choroidal contribution being considerably greater.^{6,17,18} In RPE cells, melanosomes are predominantly located in the apical and midportions of the cell. The high AF in the fovea observed with NIR-AF imaging of normal retina corresponds to the area of more intense melanization seen in SW-AF and color images.⁶ This effect is probably a result of the melanosomes being arranged cylindrically within the taller narrower RPE cells of the macula, rather than there being more melanosomes per cell.¹⁹ At the position of the intense NIR-AF band observed in the RP cases presented here, a change in melanin concentration seems unlikely. Spatial differences in the NIR-AF signal observed in RP cannot be attributed to differential loss of RPE, as we found no evidence of RPE atrophy in SD-OCT scans; we found neither a reduction in the reflectivity band attributable to the RPE-Bruch's complex nor an increase in choroidal reflectivity that would accompany atrophy. It has previously been suggested that RPE lipofuscin may also contribute to NIR-AF; however, we have not been able to generate an NIR-AF signal from the lipofuscin bisretinoid A2E when excited at 787 nm (Sparrow JR, Duncker T, Zhou J, unpublished data, 2012). On the other hand, this observation does not eliminate a contribution from other lipofuscin fluorophores.²⁰ The latter experiments also do not exclude the possibility that melanolipofuscin complexes or oxidized melanin contribute to AF. Oxidized melanin has been shown to fluoresce when excited at 479 nm.⁷ Because the bisretinoid compounds of lipofuscins do not absorb at 787 nm, lipofuscin is also unlikely to attenuate NIR-AF by absorption.

Melanin is well known to exhibit fluorescence emission under near-infrared light excitation.²¹ Although most biological pigments exhibit distinct absorption spectra, melanin has a broadband absorption band and it has been debated as to whether the absorption profile is attributable to scattering in addition to electronic properties.²² NIR photons are subject to multiple scattering and absorption and at near-infrared wavelengths, small cellular organelles, such as melanosomes, play a more important role in multiple scattering of light.^{23,24} Thus, it is interesting to consider the possibility that in lipofuscin-laden RPE cells wherein melanosomes are displaced

by the increased fractional volume of lipofuscin, the latter may not directly contribute to NIR-AF but may modify the NIR-AF properties of the neighboring melanin. For instance, perhaps regions of densely packed melanin can have low AF because of quenching of the fluorescence emission by secondary absorbance.¹⁸ Conversely, the separation of melanosomes by the interspersed increased numbers of lipofuscin granules (which do not absorb the NIR wavelengths) may facilitate release of the fluorescence emission that is detected as enhanced NIR-AF.

Although SW-AF imaging has become a standard of care in many retinal clinics, NIR-AF is less commonly used. Concerns have been raised about long-term effects of short-wavelength light in patients with retinal disorders.^{25,26} For this reason and because of patient comfort and cooperation, NIR-AF imaging has advantages, especially for children and photophobic patients. Among the three observers in this study, agreement was excellent for measurements in SW-AF and NIR-AF images, as shown by the ICCs (see Table 2). The slight trend toward stronger agreement among observers for measurements within NIR-AF images may be explained by the higher contrast between areas of low and high AF in NIR-AF. The higher contrast between the NIR-AF signal within the ring and outside the ring may help to estimate the extent of the ring and allow an approximation of remaining healthy retina, especially in cases where the SW-AF ring is quite faint.

In conclusion, NIR-AF may have advantages for clinical use, and in addition it could unveil a process that precedes the formation of fluorophores that lead to the SW-AF signal.

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