Supplementary Data

Quantitative Fundus Autofluorescence in Healthy Eyes

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A. Instrument Uniformity Measurements on Patterns

B. Horizontal and Vertical Asymmetries in AF Images of Eyes

C. Effect of Ocular Pigmentation on Reflectance and Autofluorescence

A. Instrument Uniformity Measurements on Patterns

To assess field uniformity of the Spectralis used in this study, we imaged square fluorescent targets located at about 30 cm from the scan pupil of the camera. We averaged 4 images, each with the target rotated by 90° from the previous image, to eliminate eventual non-uniformities in the target. Mean GL's measured in all segments and in some areas at the edges and corners of the image were corrected for the zero-GL and expressed in percent of the GL's at the center of the image. Mean normalized GL's for 4 independent uniformity tests carried out over the course of the project (Fig. S1) describe the (non) uniformity function U. In addition to the decrease in signal with increasing eccentricity from the center of the image, there are distinct right to left and top to bottom asymmetries, with higher values at the right and top compared to the left and bottom, respectively.

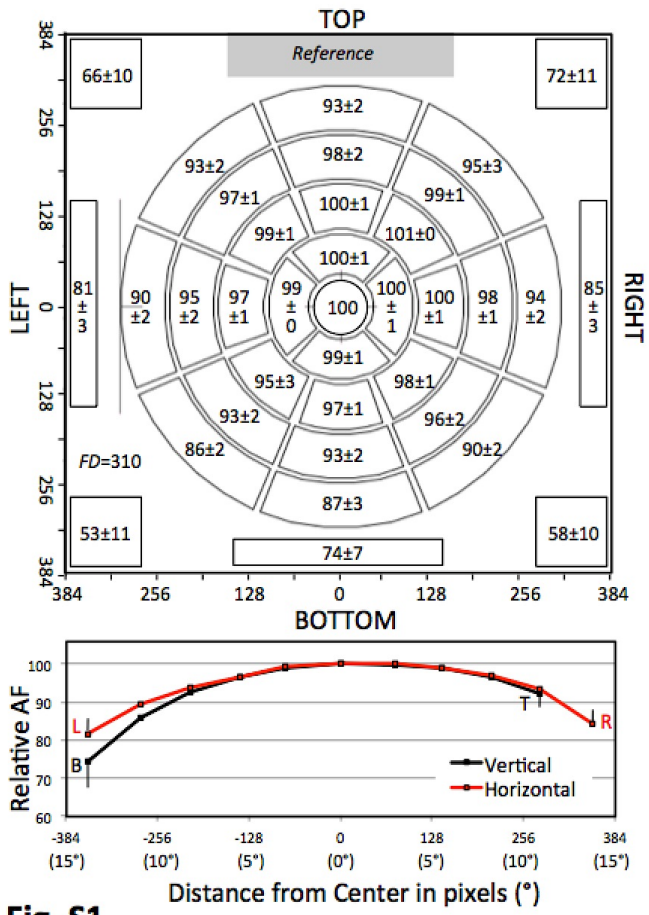

Fig. S1

To provide a measure of the horizontal and vertical asymmetries, we calculated the ratios U_{Right}/U_{Left} and U_{Top}/U_{Bottom} for the data from the test target (Table S1).

Table S1. Fluorescent Target: Median Uniformity Ratios (IQR: Inter Quartile Range)

n=4	Inner Ring	Middle Ring	Outer Ring
U_{Right}/U_{Left}	1.02 (0.01)	1.03 (0.01)	1.04(0.03)
U_{Top}/U_{Bottom}	1.02 (0.02)	1.04 (0.02)	1.07(0.02)

All ratios tend to be different than 1 (Wilcoxon Sign Test; p=0.07).

The ratio U_{Top}/U_{Bottom} showed a tendency to be larger than the ratio U_{Right}/U_{Left} (Wilcoxon Pair test, Z_4=2.0, p=0.07) but only at the eccentricity of the outer ring.

B. Horizontal and Vertical Asymmetries in AF Images of Eyes

For AF images of eyes, one cannot estimate the uniformity function U because the distribution of lipofuscin (altered by light absorption of vessels, macular pigment and RPE melanin) is not known a priori. Instead one can only derive ratios of the function U along the horizontal and vertical lines through the fovea.

We assessed the *horizontal asymmetries* by comparing AF images from the left and right eyes of 97 subjects (manuscript: Table S1) since it can be assumed that the distribution of lipofuscin is the same in both eyes. The qAFs of all segments were normalized with qAF_8 of the respective eye (qAF_8 is the mean of the qAFs for the 8 segments of the middle ring (manuscript: Fig. 1) to minimize eventual differences affecting the entire image. These normalized values M=qAF/qAF_8 can be expressed as the product of the lipofuscin distribution (L) and the instrument (non) uniformity function (U). For the temporal (T) and nasal (N) side of each eye and assuming that a unique uniformity function (Fig. S2), we have:

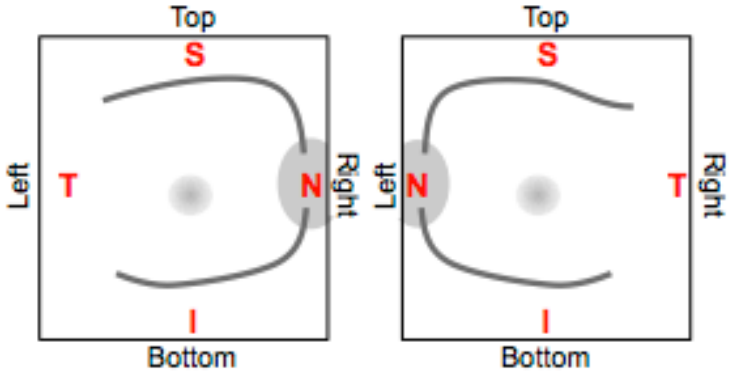

Fig. S2

$$M_{OD,T} = L_T \times U_{Left}\ ;\ M_{OS,T} = L_T \times U_{Right}\ ;\ M_{OD,N} = L_N \times U_{Right}\ ;\ M_{OS,N} = L_N \times U_{Left} \qquad \text{(S1)}$$

Combining the 4 equations we derive the ratio U_{Right}/U_{Left} by eliminating L_T and L_N:

$$\frac{U_{Right}}{U_{Left}} = \frac{1}{2} \times \left[\frac{M_{OS,T}}{M_{OD,T}} + \frac{M_{OD,N}}{M_{OS,N}} \right] \qquad \text{(S2)}$$

The ratios $M_{OS,T}/M_{OD,T}$ and $M_{OD,N}/M_{OS,N}$ were not significantly different from each other (Wilcoxon paired test; Z_{96}>0.43, p>0.6), and they were averaged to minimize measurement error. The ratio lipofuscin level between the temporal and nasal side (L_T/L_N) was also calculated from Eqs. S1 by eliminating U_{Left} and U_{Right}:

$$\frac{L_T}{L_N} = \frac{1}{2} \times \left[\frac{M_{OS,T}}{M_{OD,N}} + \frac{M_{OD,T}}{M_{OS,N}} \right] \quad \text{(S3)}$$

Ratios L_T/L_N and U_{Right}/U_{Left} did not correlate with each other (r=0.11, p=0.3), indicating effective separation of the lipofuscin and uniformity distributions along the horizontal through the fovea. The median ratios U_{Right}/U_{Left} and L_T/L_N are given in Table S2 and a histogram comparing the distribution of both ratios is shown in Fig. S3.

Table S2. Comparison of Left and Right Eyes: Median Ratios (IQR)			
n=97	Inner Ring	Middle Ring	Outer Ring
U_{Right}/U_{Left}	1.04 (0.05)	1.06(0.06)	1.06 (0.10)
L_T/L_N	1.04 (0.05)	1.15(0.14)	1.81(0.36)

All ratios are significantly larger than 1 (Wilcoxon Sign Test; p<0.0001).

Uniformity: The ratio U_{Right}/U_{Left} did not correlate significantly with age, average focus or average foveal-disc distance (FD). However, a negative correlation was found with qAF_8 for the ratios of the middle and outer rings (Spearman; r_{95}<-0.23, p<0.02). This may be the result of the variation of the no-laser zero level across the field or other small non-linearities. The variability in the ratio U_{Right}/U_{Left} indicates that, in addition to instrumental non-uniformities, camera alignment and focus may also play a role.

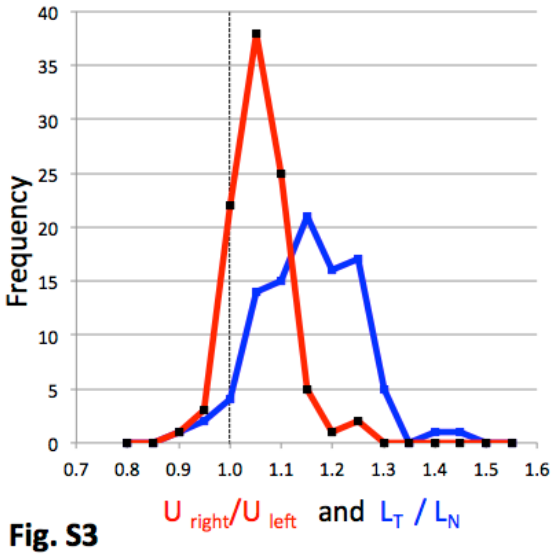

Fig. S3

Lipofuscin: For the middle ring, the ratio L_T/L_N was significantly larger than the ratio U_{right}/U_{left} (Wilcoxon paired: Z_{96}=6.5, p<0.0001). The high value of L_T/L_N for the outer ring results from the occasional presence of a peripapillary crescent (low L_N, the outer ring is tangent to disc edge). The ratio L_T/L_N was inversely correlated with the refraction (r=-0.21, p=0.03) and showed a tendency to be higher in females than in males (t=1.93, p=0.06). This suggests that asymmetries in lipofuscin distribution may be more accentuated in myopes and in females.

We assessed *vertical and horizontal asymmetries* by comparing, in 6 eyes (3 subjects), images acquired with the head tilted clockwise and counterclockwise. This yielded fundus images that were rotated by 70-80° in either direction with reference to the camera (Fig. S4). The angular position of the raster in Image 2 was then adjusted such that its segments overlaid the same retinal areas as for the segments in Image 1 (symbols ▲▼❍ ☐ indicate corresponding segments). The qAFs of all segments were normalized by qAF_8 (mean qAF in the middle ring) to minimize eventual differences affecting the entire image.

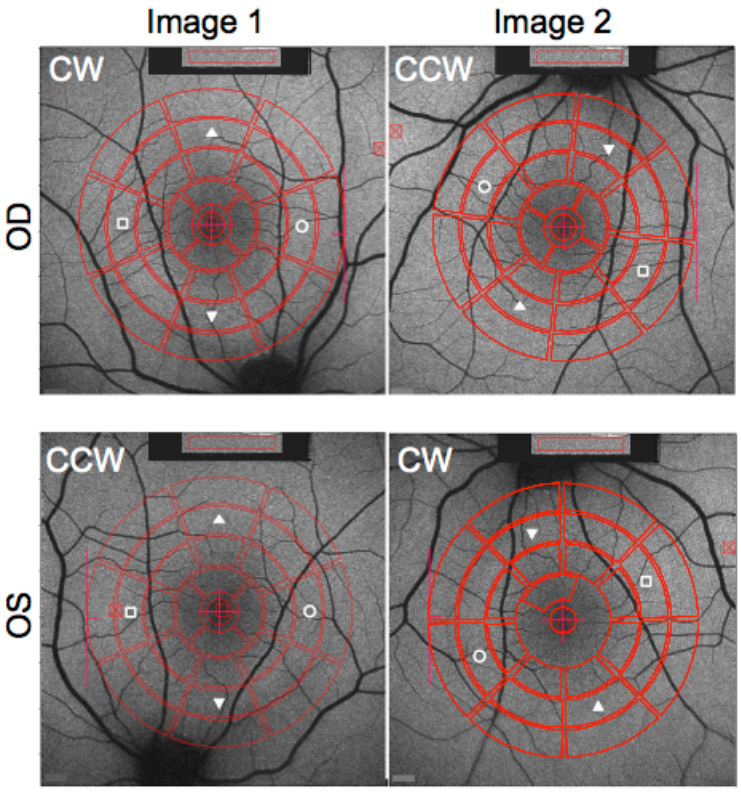

Fig. S4

For each eye, comparisons were made between the corresponding segments of Image 1 and 2. Since the retinal areas are the same, ratios from opposing segments can only be explained by asymmetries in the instrument uniformity U. The U_{Top}/U_{Bottom} ratios were derived as the average of the ratios obtained for the ▲ segments (image1/image 2) and ▼ segments (image2/image1). Similarly, the U_{Right}/U_{Left} ratios were obtained for the segments with open symbols (❍ and ☐). These comparisons were repeated for segments in the inner and outer rings (Table S3).

Table S3. Head Tilt Experiments. Median Uniformity Ratios (IQR)			
n=6	Inner Ring	Middle Ring	Outer Ring
U_{Right}/U_{Left}	1.03 (0.04) $^{(0.07)}$	1.03 (0.07) $^{(0.05)}$	1.04 (0.08) $^{(0.05)}$
U_{Top}/U_{Bottom}	1.06(0.02) $^{(0.03)}$	1.09 (0.05) $^{(0.03)}$	1.14 (0.03) $^{(0.03)}$

Superscript: p-values for difference from 1 (Wilcoxon Sign Tests).

The U_{Top}/U_{Bottom} ratios were larger than the U_{Right}/U_{Left} ratios for all 3 rings (Z_6=2.2, p=0.04). Furthermore, the ratios U_{Top}/U_{Bottom} and U_{Right}/U_{Left} correlate positively with each

other for the middle and outer rings (Spearman. ρ_4>0.89, p<0.02) but not for the inner ring (ρ_4>0.6, p=0.2).

Summary. The results for the instrumental asymmetries are summarized in Fig. S5. All asymmetry ratios between segments at opposite sides of the fovea (center of image) increase with increasing eccentricity of those segments.

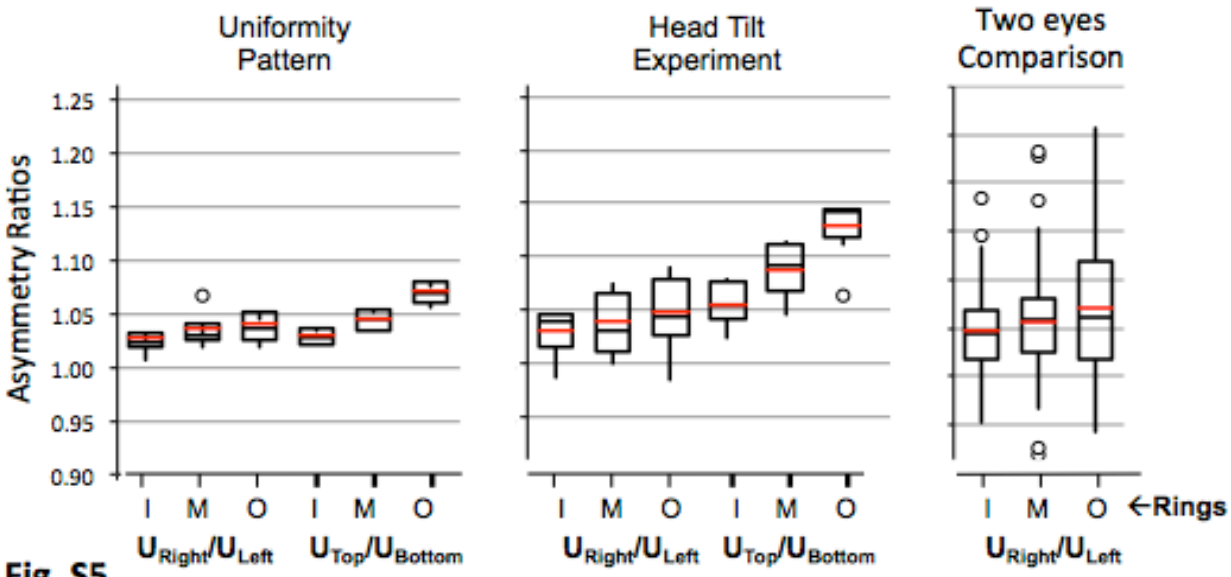

Fig. S5

The data for the uniformity pattern tests and that for the head tilt experiments are not different for the U_{Right}/U_{Left} ratios (Mann-Whitney U Test. Z<0.42, p>0.7), but the U_{Top}/U_{Bottom} ratios are larger in the case of the head-tilt experiment (Z=2.1, p=0.03). The U_{Right}/U_{Left} ratios derived from comparison of left and right eyes in 97 subjects reveal a larger variability than that observed in the head tilt experiments for 3 subjects. This may be the result of by misalignment and slight defocus during the acquisition of AF images.

In conclusion, asymmetries caused by misalignment and slight defocus affect to various degrees the uniformity in the AF images. However, the variability associated with misalignment is smaller than the variability of the lipofuscin distribution at least along the horizontal line through the fovea (Fig. S3). Thus, the effect of instrumental non-uniformities compounded by misalignment may not play a dominant role in our AF images.

C. Effect of Ocular Pigmentation on Reflectance & Autofluorescence

Fundus reflectance is strongly affected by choroidal pigmentation, particularly in red light. Fig. S6 shows mean fundus reflectance spectra acquired in a previous study for whites (W, n=32, light or dark eyes) and for non-whites (NW, 3 blacks and 1 Asian).[1, 2] Mean age of these groups was not significantly different (Mann-Whitney; Z=0.44, p=0.6). The numbers at the top of Fig. S6 are p-values for the difference between the 2 groups at each wavelength (Mann-Whitney). Fundus reflection for wavelengths below 580 nm is dominated by reflections at the photoreceptors, the RPE and Bruch's membrane, since light penetration in the choroid is limited by its strong absorption by blood. The sharp rise in reflectance above 580 nm is caused by the sudden decrease in absorption by choroidal blood. The rise in reflectance observed in non-whites is less than in whites due to the higher melanin absorption in the choroid of non-whites.[3]

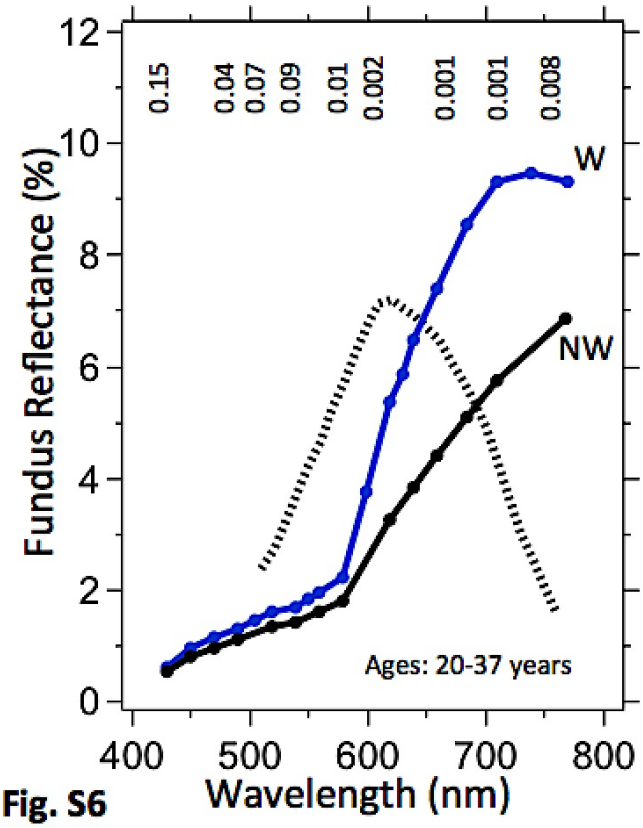

Fig. S6

Autofluorescence from the RPE is emitted over a broad spectral band (Fig S6, interrupted line). In addition to fluorescence emitted directly towards the detection pupil (Fig. S7, arrow a), fluorescence is also emitted towards the deeper layers, reflected by the choroid and transmitted by the RPE (arrow b). Furthermore, excitation light is also transmitted by the RPE and reflected by the deeper layers to further excite RPE lipofuscin from the basal side of the cell (arrow c). The total fluorescence measured at the detection pupil is

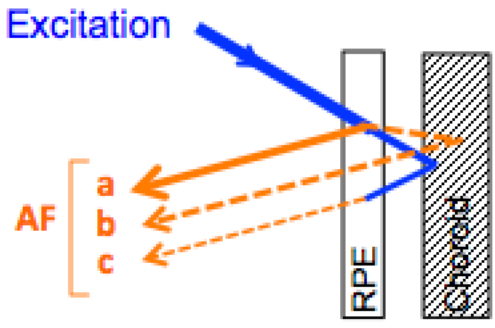

Fig. S7

thus the sum of (a), (b) and (c). Contributions (b) and (c) are both affected by pigmentation in the stroma and could result in the observed decrease in qAF for subjects with a higher degree of pigmentation.

To estimate the relative contribution of (b) and (c), we express the total fluorescence $AF_{(a+b+c),\lambda}$ at λ as:

$$AF_{(a+b+c),\lambda} = [1+\tau_\Lambda \times R_\Lambda] \times AF_{a,\lambda} \times (1+\tau_\lambda \times R_\lambda) \quad \text{(S4)}$$

where $AF_{a,\lambda}$ is the fluorescence emitted directly towards the pupil and the indices Λ and λ refer to the excitation and emission wavelength, respectively. The square parenthesis is proportional to the excitation of RPE lipofuscin (τ_Λ is the transmission of RPE lipofuscin and R_Λ is the reflectance of the deeper layers, both at Λ). It is assumed that the fluorescence is the same when the RPE lipofuscin is excited from the apical or basal side of the cell. The round parenthesis indicates by how much the fluorescence is increased by reflection of the emitted light at each λ (τ_λ is the transmission of RPE lipofuscin and R_λ is the reflectance of the deeper layers, both at λ). The reflectance spectrum of the deeper layers (*R)* can be computed from the measured spectrum by using a mathematical and optical model of the fundus. [3, 4] Instead, we assumed here that *R* is equal to the reflectance of the fundus itself and that $\tau_\Lambda \approx 1$ and $\tau_\lambda \approx 1$. Thus, the contributions (b) and (c) will be overestimated since *R* is lower than the total fundus reflectance and since $\tau_\Lambda < 1$ (τ_λ is probably close to 1, since the emission occurs outside the absorption range of lipofuscin).

We assume that the amount of RPE lipofuscin is identical in whites and non-whites or that the fluorescence $AF_{a,\lambda}$ integrated over the spectral band of the barrier filter (505-710 nm) is the same in both groups and equals 100 arbitrary units. After integration of Eq (4) over the emission spectrum, we find the total fluorescence $AF_{(a+b+c)}$ is given by:

$$AF_{(a+b+c),} = [1+\tau_\Lambda \times R_\Lambda] \times \sum_{505}^{710} AF_{a,\lambda} \times (1+\tau_\lambda \times R_\lambda) \times \Delta\lambda \quad \text{(S5)}$$

Using the fundus reflectance and the AF emission spectra of Fig. S6, we calculated $AF_{(a+b+c)}$ for the 2 groups (Table S4).

Table S4. Autofluorescence (arbitrary units)		
	Whites	Not-Whites
Total fluorescence $AF_{(a+b+c)}$ (Eq. 5)	106.8	104.5
Fluorescence AF_a (for equal amount of RPE lipofuscin)	100.0	100.0
Fluorescence contributions AF_{b+c}	6.8	4.5
Contributions AF_{b+c} in % of total fluorescence	6.4%	4.3%

Thus the contribution of reflected excitation and emission light to the total fluorescence is at most 6.4% and 4.3% for whites and non-whites, respectively. However, the ratio of total AF in whites and non-whites is 106.8/104.5=1.02, a value much smaller than the factor 20.0/14.5=1.38 predicted by the model for the ratio between whites and blacks (manuscript: Table 2).

In conclusion, although reflected excitation and emission light contribute up to 4-6% to the total AF signal, it is unlikely that these reflections play a significant role in the differences in fundus autofluorescence observed in different race/ethnicity groups.

References

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[2] F.C. Delori, S.A. Burns, Fundus reflectance and the measurement of crystalline lens density, J Opt Soc Am A, 13 (1996) 215-226.
[3] F.C. Delori, K.P. Pflibsen, Spectral reflectance of the human ocular fundus, Appl. Opt., 28 (1989) 1061-1077.
[4] J. van de Kraats, T.T.J.M. Berendschot, D. van Norren, The pathways of light measured in fundus reflectometry, Vision Res., 36 (1996) 2229-2247.