Fundus Autofluorescence in the *Abca4*$^{-/-}$ Mouse Model of Stargardt Disease – Correlation with Accumulation of A2E, Retinal Function and Histology

- Supplementary figures and tables -

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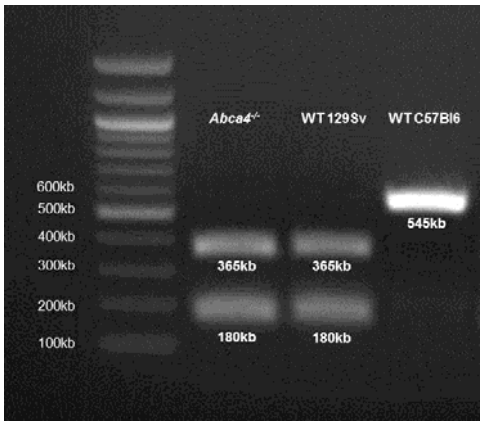
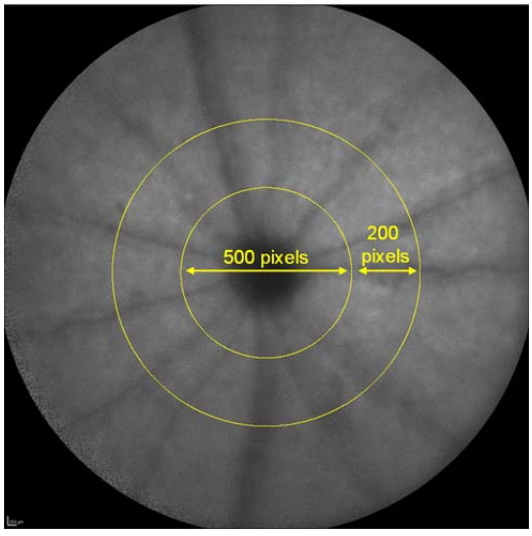
Supplementary figure 1: RPE65 genotyping



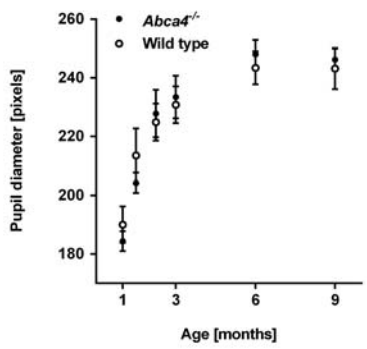
Rpe65 was sequenced by the PCR restriction fragment length polymorphism method according to Kim et al.[1] Genomic DNA was extracted from ear biopsies and PCR-amplified with the forward 5'-ACCAGAAATTTGGAGGGAAAC-3' and reverse 5'-CCCTTCCATTCAGAGCTTCA-3' primers. The Leu-450 variant introduces a Mwo1 restriction site in the resulting 545-bp product, leading to 180 and 365 bp products. *Abca4*$^{-/-}$ mice (129S4/SvJae-*Abca*^{4tm1Ght}) and wild type controls (129S2/SvHsd) showed presence of the Leu-450 variant which is associated with a higher A2E-accumulation compared to the Met-450 variant. The latter is present in the C57BL6 strain which is shown for comparison.

Supplementary figure 2: Image analysis



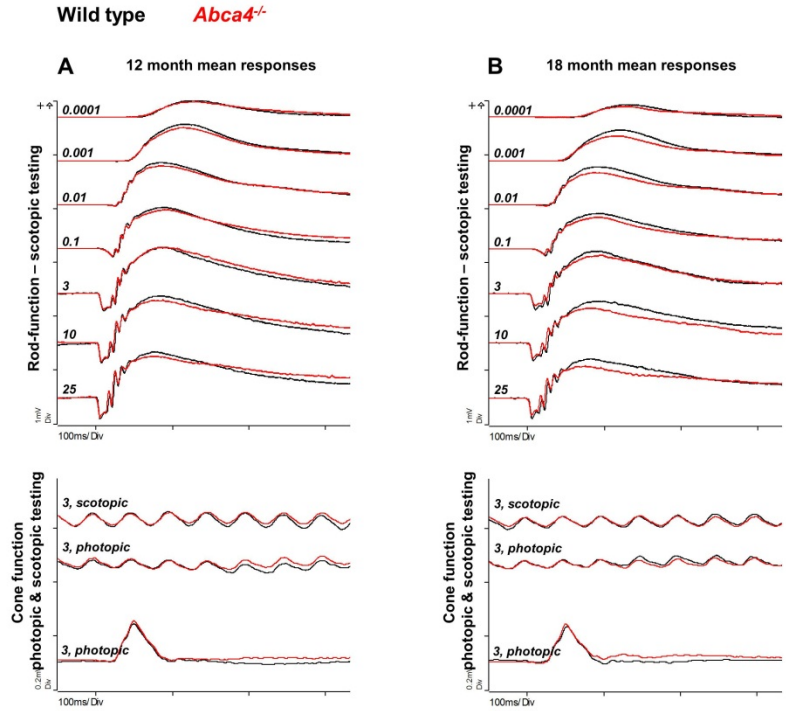
The mean grey level on mouse fundus autofluorescence images (unprocessed, 1536x1536 pixels) was measured within a ring shaped area (delineated by the two yellow circles) between 250 and 450 pixels eccentricity from the optic disc center using ImageJ software (Version 1.43, National Institute of Health, http://rsb.info.nih.gov/ij). The midperipheral area of the fundus was chosen to avoid influence of peripheral shadowing and the optic disc with the surrounding vascular crowding on the measured grey value.

Supplementary figure 3: Pupil width in *Abca4*$^{-/-}$ and wild type controls.



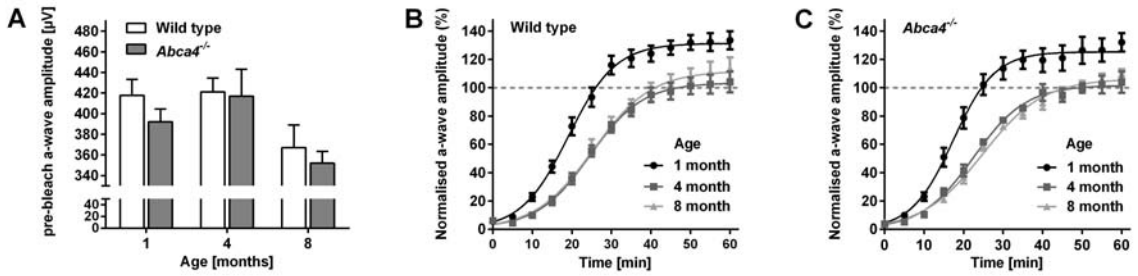
The pupil diameter (mean±SD), which strongly influences autofluorescence intensity measurements in mice,[2] increased with age ($p<0.001$) but was not different between strains (2-way ANOVA).

Supplementary figure 4: Electroretinography responses in *Abca4*$^{-/-}$ and wild type mice aged 12 and 18 months.



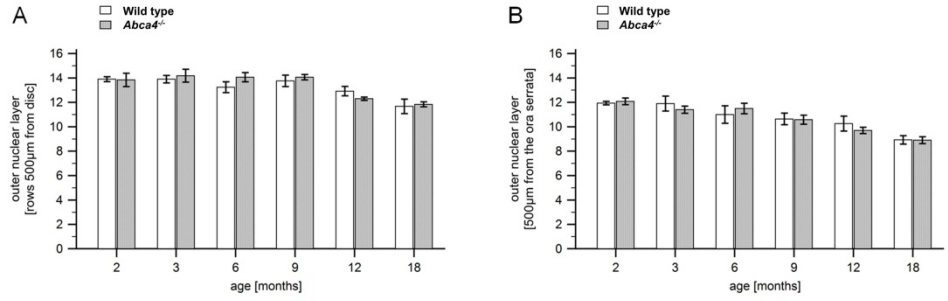
Mean responses (full range of mean traces) in animals aged 12 months (A; n=8 *Abca4*$^{-/-}$; n=6 wild type) and 18 months (B; n=5 *Abca4*$^{-/-}$; n=4 wild type). Slightly lower a- and b-wave amplitudes are present in the 18 month old *Abca4*$^{-/-}$ mice compared to wild types. There is no difference in cone function. Numbers in italics indicate flash intensity in cd.s/m^2.

Supplementary figure 5: Dark adaptation kinetics



A: Before the photobleach, dark adapted a-wave amplitudes were similar between *Abca4*$^{-/-}$ and wild type (WT) animals at all ages tested. B,C: The mean scotopic a-wave amplitude (±SEM) relative to baseline before a photobleach is shown. Dark adaptation after the photobleach was slower in 4- and 8-months-old animals compared to 1-month-old mice. This age-effect was observed in both, WT (B) and *Abca4*$^{-/-}$ (C) mice. n=5-7 in each group

Supplementary figure 6: Quantitative analysis of the photoreceptor layer

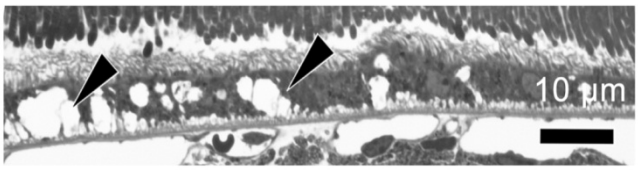


age [months]	2	3	6	9	12	18
2	-	1.0	1.0	0.03	0,03	<0.001
3		-	1.0	1.0	<0.01	<0.001
6			-	1.0	0.14	<0.001
9				-	0.02	<0.001
12					-	0.54
18						-

age [months]	2	3	6	9	12	18
2	-	1.0	1.0	1.0	<0.001	<0.001
3		-	1.0	0.3	<0.01	<0.001
6			-	1.0	0.08	<0.001
9				-	1	<0.01
12					-	0.32
18						-

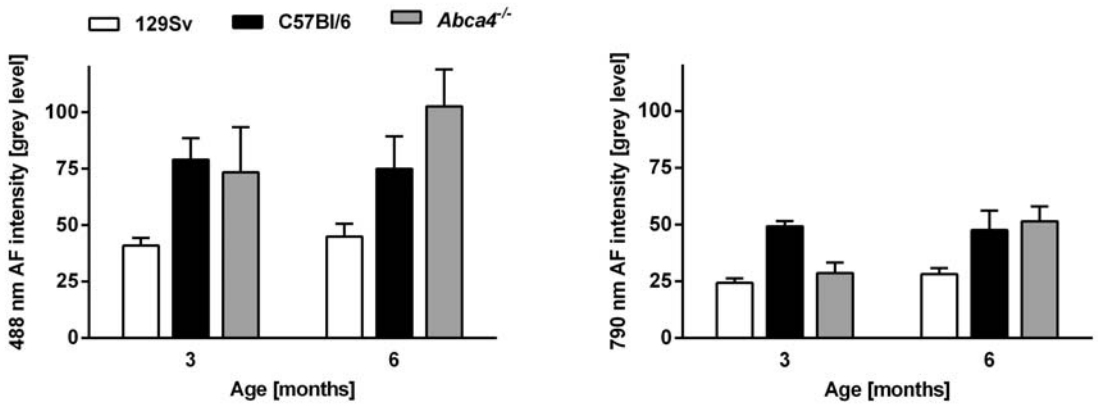
Age-related loss of photoreceptors in the central (A) and peripheral (B) retina of *Abca4*$^{-/-}$ and wild type control mice. At all ages, there was no significant difference in photoreceptor layers (mean ± SEM) between the two strains in the central and peripheral retina. However, there was a significant change with age which was most pronounced after 9 months of age (tables, 2-way ANOVA). Similar results were found in the midperipheral retina 1000µm from the disc (data not shown). n=5 for each data point, except n=4 for 18 months old wild types.

Supplementary figure 7: Vacuolization of RPE in the *Abca4*$^{-/-}$ mouse.



Vacuolization of RPE cells is indicated by arrowheads in the RPE of an 18 month old *Abca4*$^{-/-}$ mouse (bright light microscopy of a semithin section).

Supplementary figure 8: Difference in fundus autofluorescence intensity in different wild type strains compared to *Abca4*$^{-/-}$ mice.



Comparison of 488 nm (left) and 790 nm (right) autofluorescence (AF) intensity in C57BL/6 wild type (WT) mice with *Abca4*$^{-/-}$ and WT control mice of the same background strain (129Sv). Fundus AF intensity was higher in C57BL/6 WT compared to 129Sv WT mice, although C57BL/6 have the Met-450 variant in *RPE65* which is associated with less A2E-accumulation compared to the Leu-450 variant present in the 129Sv strains used (A2E-levels were not determined in C57BL/6 mice for this experiment).[1, 3] Note that the AF levels for both excitation lights do not change considerably between 3 and 6 month-old WT animals of both background strains, while there is a substantial increase in *Abca4*$^{-/-}$ mice. The difference in AF intensity between the two WT strains is in line with previous post-mortem data.[3]

Supplementary table 1: Details of the ERG protocol.

Flash intensity [cd.s/m^2]	Repetitions	Inter-stimulus interval [sec]	interval after intensity step [sec]	
0.0001	10	5	20	Scotopic testing
0.001	10	5	20	
0.01	10	5	20	
0.1	5	20	60	
3	5	20	60	
10	5	20	60	
25	5	20	120	
3	20 traces	20Hz flicker		
Pre-exposure: steady full-field white background illumination (30 cd/m^2) for 10 min				
3	10	0.5		Photopic testing
10	10	0.5		
3	20 traces	20Hz flicker		
10	20 traces	20Hz flicker		

The scotopic dose-response curve results of the three highest intensities were not averaged and only the first flash response was analyzed, because incomplete recovery between stimuli was noted.

Supplementary table 2: Details of ERG protocol to test recovery of dark-adapted function.

Flash intensity [cd.s/m^2]	Repetitions	Interval between sets [sec]	interval after intensity step [sec]	
0.001	3, applied as set	120	1-2 (manual)	After ≥6 hours dark adaptation
10				
Photobleach (400 cd/m^2) for 30 sec				
0.001	1	300	1-2 (manual)	Dark re-adaptation
10				

The results of the three baseline measures were not averaged and only the first flash response was analyzed because incomplete recovery between set of stimuli was noted.

Supplementary Table 3: Quantitative measurements of fundus autofluorescence

Age [months]	*Abca4*$^{-/-}$		Wild type	
	Longitudinal	Cross sectional	longitudinal	Cross sectional
3	71 [±7.5] (n=5)	73 [±20.0] (n=7)	37 [±7.4] (n=8)	41 [±3.4] (n=7)
6	112 [±5.6] (n=5)	103 [±16.3] (n=6)	50 [±8.7] (n=8)	45 [±5.7] (n=6)
9	112 [±9.6] (n=5)	108 [±11.0] (n=5)	52 [±9.6] (n=8)	59 [±10.9] (n=6)

Comparisons of data derived from longitudinally recorded animals (same data as in Figure 1 of the main manuscript) and from a separate cross sectional data set. Mean levels of fundus autofluorescence (AF) [±SD] are presented. The two different experiments confirm each other, providing evidence for the reliability of the method of quantitative analysis of fundus AF intensity in mice. Also, the data suggested that repeated assessments did not significantly modify AF levels over time.

Supplementary discussion

During the review of this manuscript, a paper with partly similar content was published by Sparrow *et al*. in the same journal (IOVS 2013; 54:2812-2820).[4] These authors aimed at correlating quantitative measures of fundus autofluorescence (AF) with quantitation of A2E and measurements of outer nuclear layer thickness. Our work includes a number of additional investigations, such as longitudinal and near-infrared AF imaging, qualitative analysis of AF images, functional testing, and extensive light and electron microscopy.

There are a number of common findings that mutually confirm each other's results. We have previously published a paper looking at factors which affect *in vivo* AF imaging in mice.[2] Sparrow *et al*. largely confirmed those findings in the first part of their results. In the second part of their results they reported that the increase in A2E concentration is steeper than the increase in AF intensity, which is similar to the findings reported in our current paper. In line with our discussion, they argued that this may point to other fluorophores contributing to the baseline fundus AF signal in mice (i.e. the signal without age-dependent accumulation of A2E). Also, the extent of AF intensity increase in *Abca4*$^{-/-}$ mice relative to wild type controls is of similar magnitude in both studies.

However, there are also some obvious differences between the two studies that merit further detailed discussion.

1. Modification of the confocal scanning laser ophthalmoscope (cSLO)

The cSLO used in the study by Sparrow *et al*. was fitted with a smaller pinhole limiting the diameter of the incident laser beam (normally 1.7 mm) and the detection pupil (normally 3.4 mm) to 0.98 mm. This should ensure equal amounts of light entering the small mouse eye (pupil size only up to ~2 mm depending on age and strain) across different animals and age groups. A small pinhole size is associated with a decreased AF signal at the same laser power setting. This, together with the overall lower A2E accumulation of the mouse strain used by Sparrow *et al*. (see below) may explain the darker appearance of their fundus AF images of *Abca4*$^{-/-}$ mice (e.g. their figure 2) compared to our observations (e.g. figures 1D, 2A). Moreover, th modification of the cSLO used by Sparrow et al. should improve the validity of associations between AF measures and quantification of A2E. However,

the reduced size of the pinhole would not correct for other age-dependent changes that might also modify fundus AF measures, such as eye size, lens absorption characteristics, or increasing contribution of other fluorophores (for example the due to the age-dependent occurrence of hyper-autofluorescent spots in albino mice).

In our study, we did not aim at providing quantitative AF measures that are comparable across different age groups. We focused on investigating differences between strains within the same age group. Comparison with age-matched controls also corrects for age-dependent parameters potentially modifying AF measures (as mentioned above) that are not corrected for by using a smaller pinhole size. We plotted ratios between age-matched *Abca4*$^{-/-}$ and wild type control animals, assuming similar changes of age-dependent parameters between strains, which we have exemplarily shown for pupil width.

A second modification of the cSLO used by Sparrow *et al*. was the use of an internal fluorescent reference, as described elsewhere [5], to correct for fluctuations in laser power and detector sensitivity and thereby improve reliability of fundus AF intensity measures. However, the test-retest variability was only slightly better in the study by Sparrow *et al*. (coefficient of repeatability ±18.6% versus ±22% using our method [2]; 95% confidence interval). This suggests that other parameters influence quantitative AF measures in mice considerably more than in humans (coefficient of repeatability ±6%),[5] where fluctuations of laser power and detector sensitivity contribute noise of similar magnitude. In case the latter parameters undergo long term changes such as a drop in laser power, a reference fluorophore for mouse fundus AF imaging might be meaningful for longitudinal measures, provided changes of the reference are less than the combined fluctuations of laser power and detector sensitivity.

2. Different mouse strain genetic backgrounds used: the importance of pigment.

Sparrow *et al*. used albino mice (*BALB/c*) whereas we used pigmented mice (*129S4/Sv*). *Abca4*$^{-/-}$ albino mice accumulate less A2E compared with pigmented *Abca4*$^{-/-}$ mice.[6] This slower accumulation of the A2E/bis-retinoids observed in ageing *Abca4*$^{-/-}$ albino mice may be due to increased oxidation of the bisretinoids in the albino eye followed by some clearance and/or due to photoreceptor degeneration with subsequently reduced bisretinoid formation. Photoreceptor degeneration in

Abca4$^{-/-}$ albino mice might occur due to high levels of all-*trans*-retinal which has been implicated in photoreceptor cell death. Formation of all-*trans*-retinal is increased due to the higher light levels in the albino eye,[6] and its clearance is reduced due to defective ABCA4 function.[7]

Sparrow *et al.* found increasing A2E levels in *Abca4*$^{-/-}$ albino mice up to the age of 8 months and declining levels thereafter, whereas AF levels continuously increased up to the age of 12 months – the latest time point reported. Compared to wild type controls, the photoreceptor layer thickness in *Abca4*$^{-/-}$ mice was similar at the age of 4 month, but was reduced in animals 8 months and older. The authors stated that "these findings indicated a relationship between RPE lipofuscin accumulation and photoreceptor cell death". In our study, there appeared to be a ceiling effect of AF- and A2E levels in pigmented *Abca4*$^{-/-}$ mice, with a steep increase up to the age of 3 months, approximating a maximum with only little additional increase thereafter. Furthermore, we found in pigmented mice that high levels of lipofuscin/A2E in the retinal pigment epithelium does not adversely affect retinal structure or function over prolonged time intervals. The extent of photoreceptor layer thinning and reduction of amplitudes on electroretinography testing in pigmented *Abca4*$^{-/-}$ mice only showed minor changes similar to the normal aging effect observed in wild type controls. Thus, we cannot confirm a relation between RPE lipofuscin accumulation and photoreceptor cell death in pigmented *Abca4*$^{-/-}$ mice.

In line with the previously reported difference between albino and pigmented mice, overall A2E levels were higher in our study compared to the levels reported by Sparrow *et al*. In accordance with the higher A2E levels in pigmented mice, one might expect higher AF levels than in albino mice. However, AF measures are not directly comparable between the two studies due to the differences in assessing AF intensity (see above). Of note, higher AF levels have recently been shown in albino *BALB/c* compared to pigmented *129S4/Sv* mice (figure 9 in [2]), which might be explained by the lack of melanin as an absorber of the excitation light. Moreover, albino *BALB/c* regularly begin to exhibit autofluorescent spots early in life [2] which may also occur in other wild type mouse strains, but at a much later time point. These spots might represent photoreceptor debris or macrophages and they influence grey levels analysis performed in quantitative AF assessment. If autofluorescent spots occur more frequently in *Abca4*$^{-/-}$ than in WT controls, this

might explain the slightly higher fold difference of AF intensity between *Abca4*$^{-/-}$ and wild type mice in the study by Sparrow *et al*. compared to our study. However, this has not been investigated or reported so far.

The increased photoreceptor loss of *Abca4*$^{-/-}$ mice compared to controls observed by Sparrow *et al*. might be explained by the higher levels of all-*trans*-retinal in the albino mouse eye, and thus might be independent from lipofuscin accumulation in the retinal pigment epithelium. Although A2E levels are higher in pigmented compared to albino mice, increased photoreceptor loss was not observed in our experiments using pigmented mice. These findings rather suggest the lack of a direct causative relation between A2E accumulation and photoreceptor loss. This might also explain the paradox observed in heterozygote albino knockout (*Abca4*$^{+/-}$) mice, which show lipofuscin/A2E-measures similar to wild type controls while at the same time have photoreceptor loss similar to homozygous knockout (*Abca4*$^{-/-}$) mice. The photoreceptor loss in *Abca4*$^{-/-}$ albino mice might be due to increased all-*trans*-retinal toxicity, while the increased lipofuscin/A2E levels would have no relevant effect on retinal structure and function. Notably, pigmented heterozygote *Abca4*$^{+/-}$ mice have A2E levels in between homozygous knockout (*Abca4*$^{-/-}$) and homozygous wild type (*Abca4*$^{+/+}$) mice, without a difference in photoreceptor degeneration between the three genotypes.[8]

Photoreceptor loss may also occur secondarily to cell death within the retinal pigment epithelium. However, the latter would be visible as qualitative changes on cSLO AF images. We have not found major defects resembling e.g. human geographic atrophy in *Abca4*$^{-/-}$ mice up to an age of 18 months, but such analysis was not reported by Sparrow *et al*. If the albino mice used by Sparrow *et al*. develop RPE atrophy earlier than pigmented mice, for instance due to an increased abundance of cytotoxic oxidation products of A2E, one would rather expect declining AF intensity with age. Continuously high AF values despite declining A2E values in aged mice might be explained by the accumulation of fluorescent debris and/or macrophages in mice with faster degeneration of the outer retina. In this case, however, corresponding fundus changes should be visible on AF images, and a linear relationship between A2E and qAF measures in older *Abca4*$^{-/-}$ mice would be unlikely.

3. Further differences between the two studies

Sparrow *et al*. did not use a contact lens because they identified a lower AF signal with use of a contact lens. The reasons for this observation may include inconsistencies of lens placement, which would also explain their more variable qAF measures when using a contact lens. Certainly, there may also be manufacturing-dependent variation between the custom-made mouse contact lenses. Light absorption by the lens material (PMMA) appears unlikely to provide a full explanation, because the spectral transmittance curves of this material routinely show >90% transmittance of light above 300 nm and would not explain a 40% decrease in AF signal intensity.

An additional reason for us to record images with a contact lens was the requirement of a very exact positioning of the mouse and alignment of the camera to achieve consistent results. In our hands, this procedure took more than 30 seconds, which is the time allowed if an image with additional bleaching for 20 seconds should be recorded within the first minute. This short time window for the set up was suggested by Sparrow *et al*. in order to avoid the considerable drop in AF measures due to incident lens opacity without the protection with a contact lens.

When recording images, Sparrow *et al*. did not use the ART mode as we did. Instead, they recorded a video consisting of 9 frames and averaged those images after reviewing each one for quality. This approach is certainly more reliable to reduce noise from blink or movement artifacts as frequently encountered in humans. We suggest that using the ART mode is also a valid approach because such artifacts very seldom occur in anesthetized mice.

Sparrow *et al*. used a custom made software program to eliminate the influence of retinal vessels on the measured grey level. This is indeed a more elaborate way of obtaining valid measures of AF derived from the RPE. Assuming vessel brightness does not vary considerably, including the dark vessels in the average grey value of the analyzed area would underestimate differences between strains. Thus, our results would rather underestimate the effects *Abca4* deficiency on fundus AF intensity measures.

References:

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