Noninvasive Imaging and Monitoring of Retinal Pigment Epithelium Patterns Using Fundus Autofluorescence - Review

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Abstract: Non-invasive imaging of the retinal pigment epithelium (RPE) using autofluorescence became recently available with the introduction of confocal laser scanning ophthalmoscopes. Fundus autofluorescence is usually excited at a wavelength of 488nm and the emitted light is detected above 500nm. This intrinsic autofluorescence was shown to derive from the lipofuscin accumulating within the RPE either with age or also due to different hereditary or degenerative diseases of the macula as e.g. age-related macular degeneration. Since a variety of macular diseases correlate with distinct RPE changes, specific patterns of autofluorescence could be evaluated within the recent years for diagnostic and prognostic reasons in those RPE-related diseases. Moreover autofluorescence can also be regarded as a monitoring tool after therapeutic applications as macular surgery or laser treatment. Other new applications try to determine macular pigment density using autofluorescence or use it to evaluate oxygen-dependent cell metabolism. This review summarizes the recent findings of autofluorescent patterns in specific diseases and therapeutic approaches and emphasizes on the tremendous potential of this novel imaging method.

Keywords: Fundus autofluorescence, retinal pigment epithelium, RPE, lipofuscin, age-related macular degeneration, macular holes, laser photocoagulation, selective RPE laser treatment, online autofluorescence.

REVIEW

The retinal pigment epithelium (RPE) is a single layer of cells between the neurosensory retina and the choroid, which itself is separated from the RPE by Bruch´s membrane. The RPE is an important structure for the maintenance of the outer blood-retina barrier, it takes part in the vitamin A circulcation, it synthesizes extracellular matrix, it transports molcecules and one of the main issues is to phagozytise the outer discs of the photoreceptors. Without the permanent RPE phagozytosis no proper photoreceptor function is possible. Degradation of the phagozytised material happens in special intracellular compartments, the lysosomes. The endproducts usually are delivered to the basal side of the RPE cells and transported by choroidal circulation. With age or due to different diseases RPE function might be incomplete and accumulation of the intralysosomal molecules, the lipofuscin, takes place [19, 41, 42, 79]. The accumulation of lipofuscin is mainly derived from the chemically modified residues of the incompletely digested photoreceptor outer segment discs [9, 42], and contains at least ten different fluorophores [16]. Thus it is believed that metabolic activity of RPE correlates with the content of lipofuscin in RPE cells [43]. Excessive accumulation of lipofuscin within the RPE may play a major role in the pathogenesis of age-related macular degeneration [4, 33, 35, 73] or other hereditary macular diseases as Best disease or Stargardt disease [1, 11, 14, 30, 44], all leading to significant loss of vision due to degeneration of RPE and consecutively degeneration of the photoreceptor layer. The intrinsic fundus autofluorescence (AF) of the RPE was shown to be derived from the lipofuscin within the retinal pigment epithelium [9, 11, 40, 70, 72, 73]. However, there are other fluorophores both anterior and posterior to the RPE cell monolayer with autofluorescent properties, even in the excitation and emission wavelength range applied here. Their intensity is; however, far below the lipofuscin derived autofluorescent signal [62].

One of the major fluorophores of the lipofuscin, N-retinylidin-N-retinylethanolamin (A2-E), which was recently identified [17, 18, 51], seem to play a major role for degradation of the RPE cell since it possesses toxic properties due to the ability of disintegration of lysosomal membranes by altering intracellular pH and phototoxic effects mediated by blue light [37, 60].

Correlating to specific diseases or to distinct levels of metabolic activity different intracellular concentrations of lipofuscin seem to be present and therefore autofluorescent activity of the RPE layer might offer specific patterns. However, due to optical limitations of the eye combined with the small size of the fluorescing elements of the RPE and strong absorption of light by the apical lying melanin granules *in vivo* imaging of AF was nearly impossible [69]. With the introduction of highly sensitive confocal scanning laser ophthalmoscopes in the 90s, imaging of AF as an index of lipofuscin and its spatial distribution over large retinal areas *in vivo* became possible [66, 72]. AF imaging was shown to provide very detailed information about the levels and distribution of lipofuscin of the RPE within the living eye [35, 36, 38, 72, 73, 74, 78]. First detailed two-dimensional images of AF within a 30° over 30° area from

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the macular area involving the optic disc and the large vessel arcades were provided by von Rückmann using a prototype of a confocal laser scanning ophthalmoscope donated by Zeiss (Zeiss Oberkochen, Germany) [72]. The *in vivo* recordings of AF show good evidence that it is derived from the lipofuscin in the RPE [3, 8, 9, 12, 13, 43, 70, 72, 73]. As shown by Delori *et al.* the spectral characteristics of *in vivo* AF are consistent with those of lipofuscin fluorophores with reference to Eldred [16]. Distribution of AF was evaluated in healthy subjects and in several macular diseases including age-related macular degeneration (AMD), hereditary diseases, macular hole formation and other RPE related diseases as e.g. central serous chorioretinopathy [71]. Especially Holz *et al.* provided excellent images of geographic atrophy, a subtype of AMD, which goes along with large areas of complete RPE atrophy concomitant with a lack of autofluorescence in those areas [36]. Thus all conditions affecting the RPE should have an influence on AF patterns. This also includes inflammation of RPE cells as in acute posterior multifocal placoid pigment epitheliopathy (APMPPE); [26] and especially biological reactions of the RPE to conventional laserphotocoagulation [25], which is widely used in ophthalmologic practice. Moreover AF changes could also be observed after selective RPE / retina laser treatment (SRT), a novel laser approach to treat RPE related diseases without affecting the neurosensory retinal layer, which will usually irreversibly destructed by conventional laser photocoagulation [21, 55, 57, 58].

For exciting AF in clinical settings nowadays the Heidelberg Retina Angiograph (HRA; Heidelberg Engineering, Germany) is the only device to obtain high quality AF images and is widely used since it was originally built for performing fluorescein angiography using the same wavelength. The HRA is a confocal scanning laser ophthalmoscope equipped with a laser source, capable of emitting laser light at four different wavelengths for different acquisition modes. AF is excited by the argon blue wavelength (488nm) used originally for fluorescein angiography. A barrier filter at a wavelength of 500nm separates the excitation and fluorescent light. The illumination beam is 3 mm in diameter and the aperture of the dilated eye is used to collect light from the posterior pole. Maximal retinal irradiation at 30° was 2mW/cm^2, well below the limits established by the American National Standards Institute [67]. The confocal detection unit employs a 400µm pinhole aperture to suppress light from below or above the confocal plane. The size of the scanning field used was 30° x 30°. Before imaging, the pupil is dilated to a diameter greater than 6mm using phenylephrine 2.5% and tropicamide 1% eye drops. The argon laser mode is used for AF image acquisition. The sensitivity of the scanning laser ophthalmoscope detector is adjusted until good quality AF images appeared on the monitor screen. A series of images has to be taken. The frame grabber of the HRA can digitize image frames at a programmable rate of up to 20 frames per second. Each frame contains 256 pixels vertically and 256 pixels horizontally. Images are digitized with 256 gray scale levels. Higher image resolutions became available within the last year. The digital images are saved for processing, where an average image from the original image series need to be created to reduce noise and to produce more detailed images for the spatial distribution of AF [72].

AF images usually are evaluated for the presence of areas of decreased or increased AF and those areas are compared with angiographic features. It is known from in-vivo and in-vitro studies that with age, a continuous accumulation of autofluorescent material occurs in the RPE [79]. At the same time, variation of AF with age increases. Thus, there is presently no possibility of defining normal age-related AF-levels [36]. Abnormal AF was defined either as an increased or decreased fundus AF signal compared with the AF outside of the lesions, the latter being referred to as normal AF, which was described earlier [66, 72]. Important for the distinction between normal and abnormal AF in the images is the spatial distribution of AF signals compared with normal AF. Usually, abnormal AF is characterized by decreased AF as derived from RPE atrophy or blockage, or due to increased AF as derived from RPE reaction. Entire areas that encompass abnormal AF including irregular elevated AF signals and patches of normal-appearing regions can be distinguished from areas with normal background AF, which is characterized by a reduced and more even AF pattern [36].

Normal fundus imaging revealed a decreased autofluorescence at the perifoveal area. The optic nerve head and the vessels are appearing dark [9, 73]. The decreased autofluorescence toward the foveal area is explained by absorption of short wavelength light by the macular pigments as lutein and zeaxanthin [72]. Moreover *in vitro* results showed less density of lipofuscin granules within this area [9, 79, 80]. The area around the vessel arcades reveals the highest intensity of autofluorescence and decreases towards the periphery [19, 20, 80]. This is consistent with histological findings demonstrating a decay of lipofuscin correlating to the density of photoreceptors toward periphery [79, 80], (Fig. **1**).

AMD is the most important macular disease in older patients leading to vision loss in the industrial nations. Usually early stages consisting of subretinal deposits called drusen and pigment irregularities were distinguished from advanced stages. Advanced AMD goes along with either dry forms as geographic atrophy or wet forms including choroidal neovascularizations or RPE detachments, which often lead to a fast and irreversible loss of vision. AF in early stages often reveals irregular areas of increased AF depending on the progress of disease [1, 35, 66, 69, 73]. The German FAM study group currently tries to define and characterize different types of AF patterns associated with those early forms of AMD [15]. Since it is known that late stages of disease progress from those early ones AF might be a helpful tool for giving valuable prognosis in individuals regarding to early AF patterns (Fig. **2**). Thus it could already be shown that enhanced perifoveolar AF might lead to an earlier and faster progression to geographic atrophy [39]. In geographic atrophy smaller patches or large areas of complete RPE atrophy are present. Significant visual loss occurs when the foveolar region is affected. As already shown by Holz *et al.*. using AF imaging the area of RPE atrophy is dark revealing complete loss fluorophores [36, 38]. Interestingly usually a smaller or broader band of

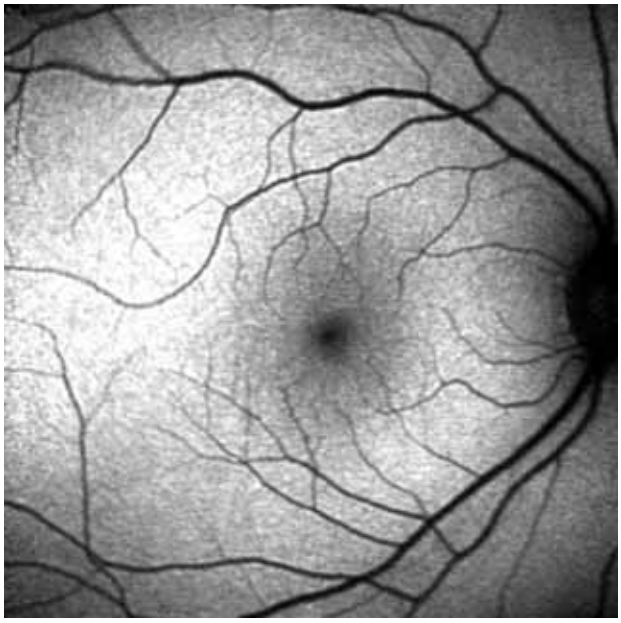


Fig. (1). Normal AF image of the macula over a field of 30° over 30°. The optic disc and the vessels appear dark. AF is enhanced around the vessel arcades and fades peripherally. In the foveolar region AF is physiologically decreased due to a lower amount of lipofuscin and blockade from the macular pigments as lutein and zeaxanthin.

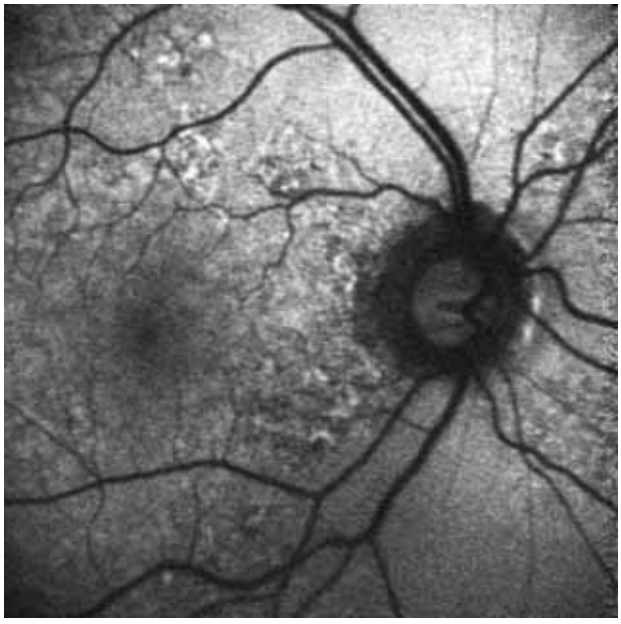


Fig. (2). AF image of early AMD consisting of drusen and pigment changes. AF revealed areas of pathological enhanced AF.

significantly increased AF in the junctional zone was found indicating a higher level of intracellular lipofuscin and thus a higher risk for consecutive cell death (Fig. **3**). Actually over time a progression of geographic atrophy was present within the area of previously increased AF [36]. This underlines the pathologic relevance of lipofuscin accumulation, it could be

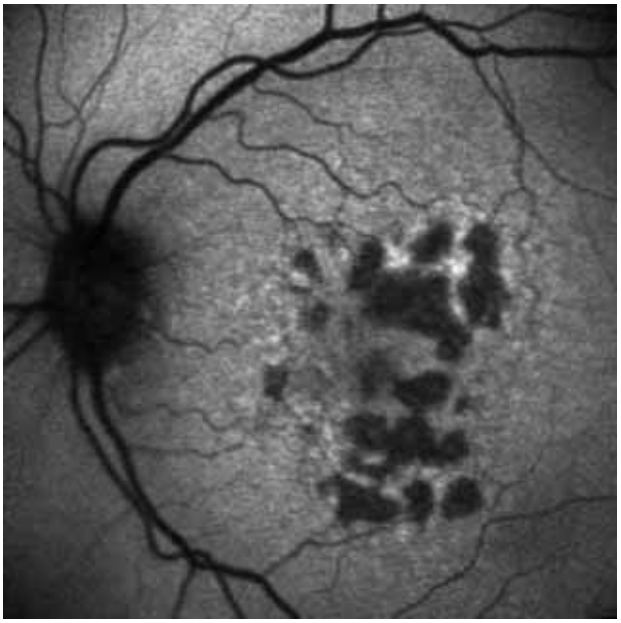
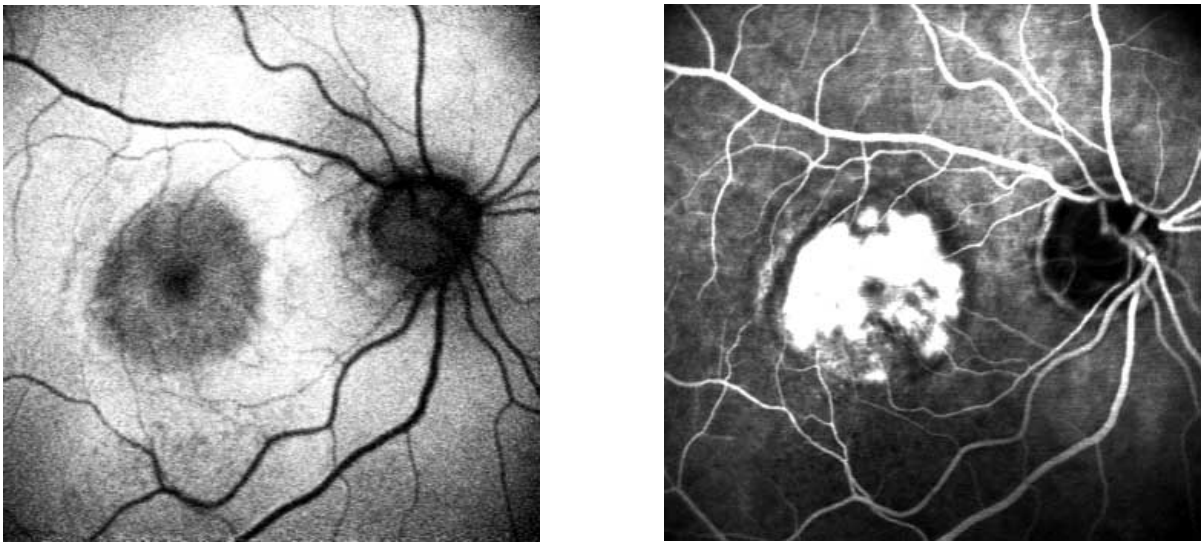


Fig. (3). AF image of geographic atrophy due to advanced AMD. In the area of RPE cell loss AF is dark suggesting complete atrophy. In the junctional zone AF is markedly increased indicating lipofuscin accumulation. Geographic atrophy will spread into the areas of increased AF.

used as a prognostic factor and might give hints for prophylactic treatment as e.g. with the new method of SRT. Today only little focus was set on the wet forms of AMD using AF imaging. Von Rückmann *et al.* described very heterogeneous AF patterns in choroidal neovascularizations (CNV), [73]. Usually these CNV´s lead to a sudden irreversible visual loss leading to macular edema, subretinal hemorrhage and consecutive scarring. CNV´s are usually diagnosed by fluorescein angiography, which distinguishes those subretinal neovascular membranes in predominantly classic or occult ones. From the pathoanatomic point of view classic membranes usually lie above the RPE and could be well demarcated in angiography whereas occult ones usually stay beneath the RPE level leading to poor demarcation [32]. Prognosis and treatment depends on these important classifications, however invasive fluorescein angiography is necessary to establish correct diagnosis and important for follow up examinations. As found in our series [22] there is a hint that the two entities of CNV might also be distinguished by AF revealing significantly decreased AF in the majority of eyes with classic CNV over the whole area of the membrane surrounded by slightly enhanced AF whereas only slightly irregular and enhanced AF was found in occult CNV [22], (Fig. **4**). These findings correlate with angiographic features and could be explained by the location of the membrane, blocking AF when lying above the RPE as in classic membranes and slightly enhance it when lying beneath the RPE as in occult ones. If these results could be approved in a larger population AF might be able to replace angiography in some cases of wet AMD.

As already confirmed by some studies AF is an interesting tool for differential diagnosis in hereditary diseases [46]. As proven by histologic studies in former



4a **4b**

Fig. (4a, b). AF image and corresponding fluorescein angiography of a classic choroidal neovascularization due to advanced AMD. Angiography revealed leakage from the neovascular membrane. Because these membranes usually lie above the RPE demarcation is enhanced also in AF showing a well demarcated blockade of AF. In the junctional zone AF is increased indicating enhanced cell metabolism (Image taken from Ref.22).

years excessive accumulation of lipofuscin is present in diseases as e.g. Stargardt or Best disease. In Stargardt disease diffusely enhanced and focally increased AF is present in ophthalmoscopically normal appearing retina (Fig. **5**). Performing fluorescein angiography the phenomenon of "dark choroid" is known since intracellular lipofuscin blocks the choroidal fluorescence during examination. AF is able to confirm the diagnosis of Stargardt due to the specific pattern making fluorescein angiography obsolete. However, AF pattern might also be very heterogeneous and current studies try to determine correlations of AF phenotype with genotypic phenotypes also to evaluate the potential of AF derived prognosis for individual disease [46]. These studies were also undertaken for evaluation of Best disease, which usually goes along with centrally increased AF intensity presenting as vitelliform lesions [75]; (Fig. **6**).

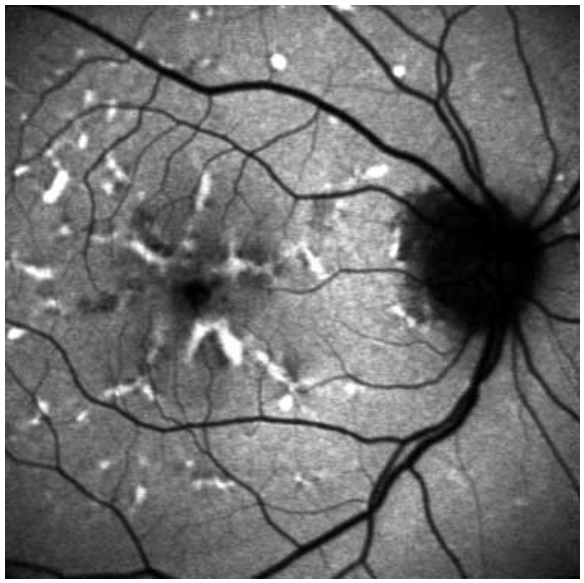


Fig. (5). AF image of Stargardt´s disease. AF is generally increased due to the pathologic accumulation of lipofuscin in early years. Moreover numerous areas of focally increased AF are seen.

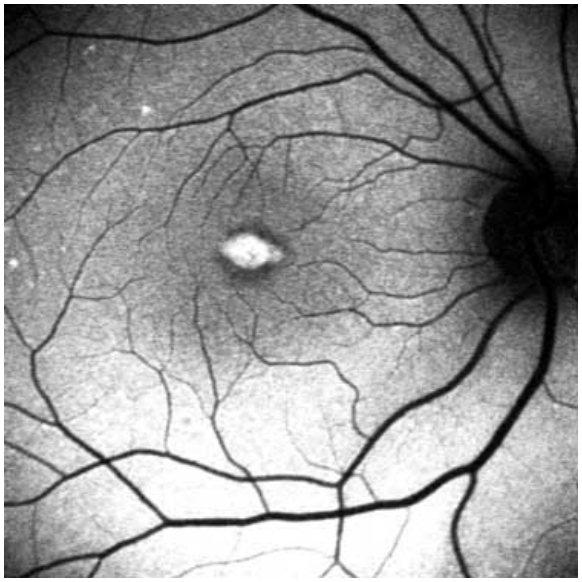


Fig. (6). AF image of Best's disease. Excessive accumulation of lipofuscin takes part in the central area leading to ophthalmoscopically seen Bull's eye maculopathy and highly increased AF. In advanced stages lipofuscin slides and a "scrambled egg" appearance occurs.

Besides degenerative and hereditary diseases also inflammatory processes could especially affect the RPE. Proper diagnosis is usually based on ophthalmoscopy and angiography; however, since RPE is directly affected a change of AF patterns might be expected. A typical disease is the idiopathic acute posterior multifocal placoid pigment epitheliopathy (APMPPE), which mainly affects young healthy male or female patients who develop a rapid loss of vision in one or both eyes secondary to multiple postequatorial, circumscribed, flat, gray-white, subretinal lesions involving the RPE [32]. The characteristic feature of the disease is the rapid resolution of the fundus lesions and the delayed remarkable return of visual function to the level of 20/30 or better [32]. Within a few days following the onset of symptoms the acute gray-white lesions begin to fade centrally. Within 7 to 12 days they are completely replaced by areas of partly depigmented RPE followed by clumping of pigment and day-to-day changes over a period of months afterwards looking like subacute lesions after photocoagulation [32]. Usually the etiology of APMPPE is unknown but it is described in association of viral infections [81]. In patients with APMPPE interestingly, the lesions could be visualized very well by demarcated increased AF, which enhance after days to weeks and than decrease again leading to RPE atrophy after one year [26]; (Fig. **7**). Increased AF seems to monitor the higher metabolism of the affected cells; however, it must remain speculative if AF is derived only from lipofuscin, which is questionable accumulating in such an excessive way only within days. It might also be possible that affected cells die and reproliferation of bystander RPE cells cover the defect leading to multilayered RPE and thus enhanced AF appear. However, as confined to AF interestingly final RPE atrophy occurs after one year, and prognosis might only be good, if initially affected areas are not in the fovea [26]. Thus AF seems to be a suitable tool for monitoring biological reactions within the RPE cell even over months to years.

As already described for classic CNV due to AMD AF patterns must not necessarily only be confined to changes within the RPE cell itself. Also specific tissue conditions outside the RPE might lead to irregular AF. Thus increased AF could be found in macular hole formation, where due to a lack of overlying neurosensory tissue, RPE fluorescence is detected at higher levels from increased transmission, while the RPE condition itself stays intact [24, 74]. On the other hand, decreased AF is physiologically found in the foveal area due to a blockage of AF from the macular pigments, e.g. as lutein preventing blue-light damage [72]. However, blockage of AF due to subretinal edema can also lead to decreased AF [21, 25].

Regarding macular holes AF could be established as an excellent diagnostic and monitoring method to non-invasively determine stages of macular holes undergoing surgery. Macular holes usually appear in older people with female prevalence leading to central vision loss. The pathogenesis includes tractional forces from the vitreous on the macular surface. Usually the diagnosis of a macular hole is performed by biomicroscopic examination. However sometimes it can be hard to differentiate between a full thickness macular hole and so-called pseudoholes. Due to tangential traction on the retinal surface or after trauma hole formation can appear at the foveolar site gaining a lack of neurosensory retina. Due to this hole formation uncovered RPE becomes apparent. Von Rückmann *et al.* first described the AF patterns of this unblocked RPE in macular hole formation leading to significantly increased AF whereas pseudoholes did not [74]. In our series 25 consecutive eyes

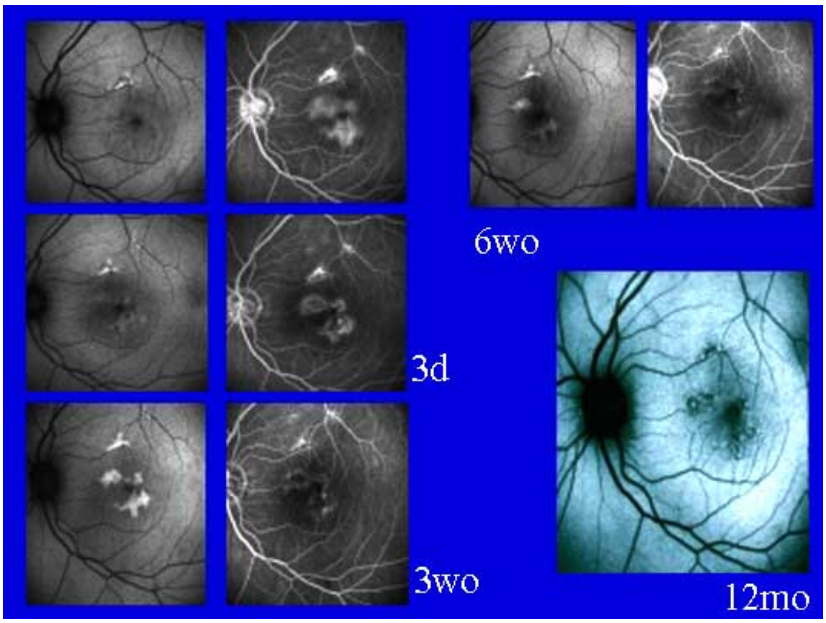
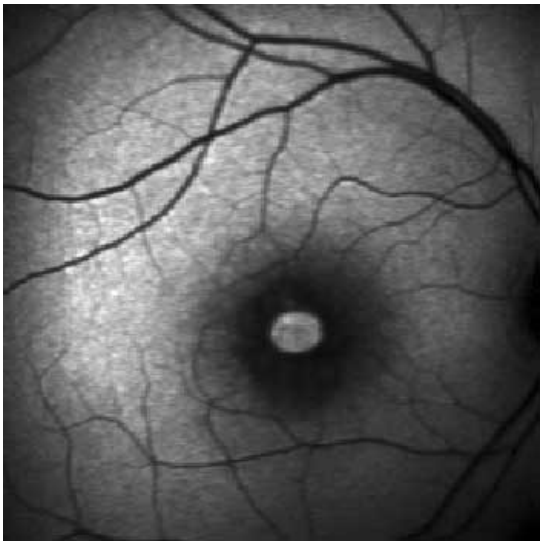


Fig. (7). AF sequence of an eye with APMPPE. Affected RPE cells demonstrate increased AF in the early phase of the disease, which is more pronounced after 3 weeks and then again fade away. Interestingly atrophy seems to occur after one year leading to significantly decreased AF in former lesions (Image adapted from Ref.26).

with idiopathic macular holes and clinically similar appearances were retrospectively evaluated [24]. In all patient s with a full thickness macular hole stage 3 and 4 AF showed marked increase of central AF (Fig. **8**) and a corresponding window-defect in fluorescein-angiography. In patients with a macular hole stage 2 AF revealed slightly irregular and enhanced AF and just a subtle window-defect in fluorescein-angiography. After successful surgery including pars-plana vitrectomy, peeling of epiretinal membranes, application of autologous concentrate of thrombocytes and gas instillation AF revealed closure of the macular hole due to a lack of increased AF (Fig. **9**). Thus AF is considered as a useful adjunct tool in macular hole surgery especially in questionable biomicroscopic findings. As already stated by von Rückmann it is possible to differentiate between full thickness macular holes and pseudoholes and postoperatively it is helpful for the estimation of the therapeutic success. In contrast to fluorescein angiography AF has the advantage of having no side effects like allergic reaction, it is cheaper and it requires less time and manpower. In our hospital AF has successfully replaced fluorescein angiography for differential diagnosis of macular holes and establishment of surgical success.

Another interesting disease demonstrating potential AF changes due to intracellular and extracellular processes is the central serous retinopathy (CSR). The disease is characterized by a shallow, round, and serous detachment of the neurosensory retina; however, small detachments of the retinal pigment epithelium (RPE) may also occur [32]. Patients usually complain about blurred vision, scotoma, micropsia or metamorphopsia. On fluorescein angiography, single or multiple focal leakage sites can be identified. CSR is known as a disease of the RPE, leading to pathologic breaks within the RPE layer and the consecutive subretinal leakage; however, the choroid seems to also be affected [34]. AF patterns in CSR were also primarily described by von Rückmann [71], showing distinct patterns in acute and chronic or healed disease. In our series [29] eyes with acute CSR revealed neurosensory retinal detachment in the central area with focal leakage points within this area. Neurosensory detachment could be underlined by optical coherence tomography clearly demonstrating the retinal elevation. Due to the subretinal fluid, which often consists of higher protein content depending on latency underlying RPE and consecutively AF is blocked (Fig. **10**). Prognosis of the disease is excellent leading to closure of the RPE defect at the leakage point and subretinal fluid diminished. AF in eyes with healed CSR showed increased AF within the whole area of previous detachment (Fig. **11**). In contrast to another study which describes an increase of AF at the site of leakage [71], we found partially increased AF only several weeks after the onset of symptoms in areas surrounding the original point of leakage, which itself showed decreased AF. It is thought that the apparent subretinal fluid from the leaking point acts as a blockade for the underlying fluorescent RPE. The pattern of markedly increased AF studies in the area of presumed former or residual neurosensory detachment might be confined to a higher metabolic activity of the RPE cell layer, leading to a stronger accumulation of lipofuscin and therefore increased AF. An explanation of this phenomenon might be a higher phagocytosis rate of debris from the subretinal space during detachment due to presumed photoreceptor dysfunction [71]. The phenomenon of increased AF after healed or in chronic CSR could partially be of great value for differential diagnosis and might be able to replace angiography in this entity [29].

AF seems not only be able to give distinct hints for diagnosis but might also be able to monitor therapeutic applications as laser photocoagulation or SRT. Monitoring of laser lesions for instance should give a better insight to the biological reaction of the tissue and might also be of



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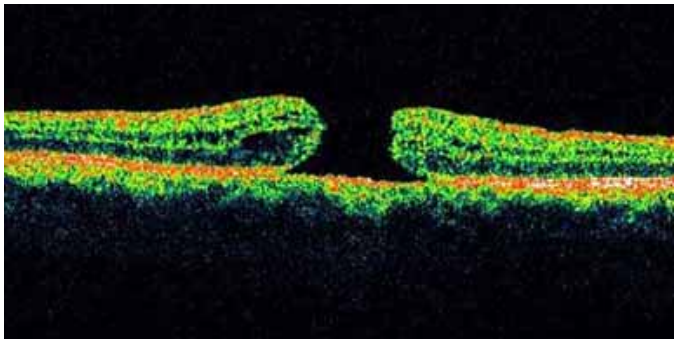


8b

Fig. (8a, b). AF image and corresponding OCT image of a full-thickness macular hole. Due to a lack of neurosensory retina bare RPE cell layer is present on the ground of the hole. This could be demonstrated by OCT and leads to a significantly increased AF with no blockage from macular pigments.

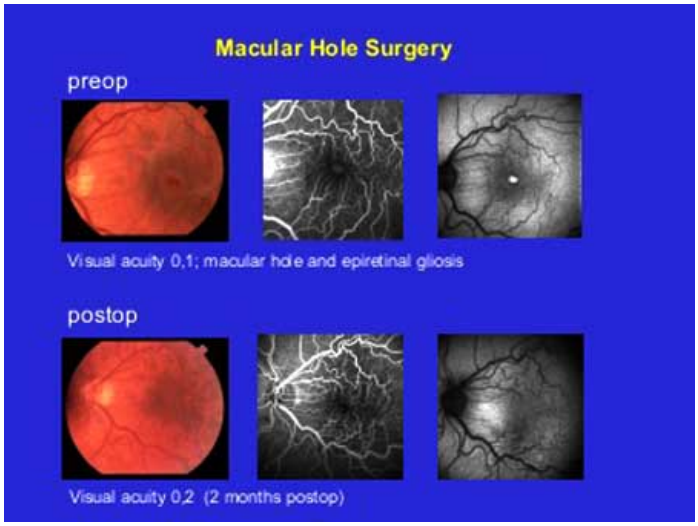
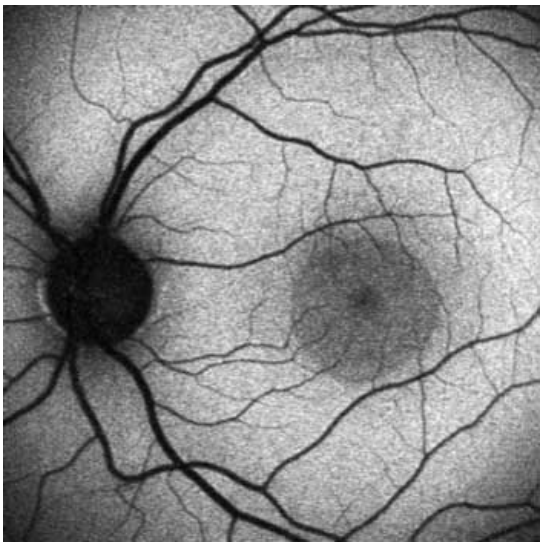


Fig. (9). Corresponding AF, angiography and fundus photograph of a macular hole before and after surgery. The preoperatively striking increased AF has gone after successful closure of the hole leading to scar formation above the level of RPE and therefore blocking the AF (Image adapted from Ref.24)

prognostic relevance. Conventional laser photocoagulation has been shown to be beneficial in a variety of retinal diseases like age related macular degeneration (AMD), diabetic maculopathy (DMP), diabetic retinopathy (DRP) or central serous retinopathy (CSR). There are several hints that the positive effect is mediated by the RPE [48, 68]. The RPE is the main target of laser energy due to its high amount of melanosomes and absorbs about 50 to 60 % of the energy applied to the retina [31]. Today conventional retinal laser treatment is performed using the continuous-wave argon laser (514 nm). Generally the exposure times are longer than 50 ms, typically 100 to 200ms. After application of the laser energy onto the retina usually an ophthalmoscopically visible grayish-white lesion results from thermal heat conduction. Histologically a destruction of the RPE, which is the primary absorption site, occurs, leading to an irreversible destruction of the outer and inner segments of the neuroretina due to thermal denaturation [2, 45, 49, 76].

The effect of laser treatment to the fundus was studied by several groups. *In vivo* it could be observed that argon laser photocoagulation of the monkey- and human fundus causes necrosis of the RPE and a detachment of the RPE from Bruch´s membrane [63, 64, 65], budding of individual RPE cells [47, 63] and a multilayered RPE formation in the area of laser irradiation by seven days after treatment [48, 50, 52, 63, 64, 65]. Histologic sections revealed that by irradiating the RPE with a conventional argon laser the whole area of the cells is destroyed and the choriocapillaris as well as the vessels of the choroid are damaged [6]. After laser photocoagulation RPE cells migrate and proliferate to cover the defect [77]. *In vivo* after mild coagulations as usually performed in macular coagulation the RPE barrier gets intact again [77]. Because AF is mainly confined to the lipofuscin within the RPE it could be supposed that alterations in the RPE due to laser destruction lead also to a change in AF behavior. It was expected that AF intensity decreases directly after laser photocoagulation due to RPE destruction with presumed damage of the fluorophores and also due to consecutive edema from the breakdown of the blood-retina barrier, which acts as a blockage of the autofluorescence signal. In fact in nearly all patients examined one hour after focal laser treatment AF was decreased in the area of laser lesions [25]. However, after one month in all lesions decreased AF changed to significantly increased AF, which was stable up to 6 months after treatment. Then lesions again changed to complete dark spots in AF after one to two years enlarging later on (Fig. **12**). In between both appearances (increased and decreased AF) mixed forms were present at about 6 to 12 months after treatment, showing a central island of increased AF surrounded by a ring of decreased AF [25]. To explain the autofluorescence decay after the laser treatment presumably three possible mechanisms are present: first RPE cells will be damaged by the laser interaction and thus leading to a acute lack of RPE correlating with decreased AF, secondarily a focal edema could act as a blockade for AF and in third place it is also known that lipofuscin AF in solution – after the RPE cell is destroyed – decreases [54]. Presumably all mechanisms act together but indicate clearly a destruction of the RPE, which is correlating to histologic findings after cw-laser photocoagulation [23]. After initial laser treatment the cell debris of damaged RPE cells and photoreceptors are phagocitized by RPE cells sliding in from the neighborhood or by macrophages originating from the choriocapillaris [55]. Abundant phagosomes can be found within these cells during this highly active period. AF then shows marked lesions of increased AF (one month up to six months). The storage granules accumulate within the RPE cells and should be responsible for the increased AF signal. However, the origin of the signal itself may also be derived from different fluorophores as from coenzymes as e.g. FAD or $FADH_2$ (personal communication Dr. Hammer, Jena, Germany) but unlikely from lipofuscin. The finally found “dark spots” indicate RPE atrophy at the lasered site. It might be supposed that due to the scarring of the overlying



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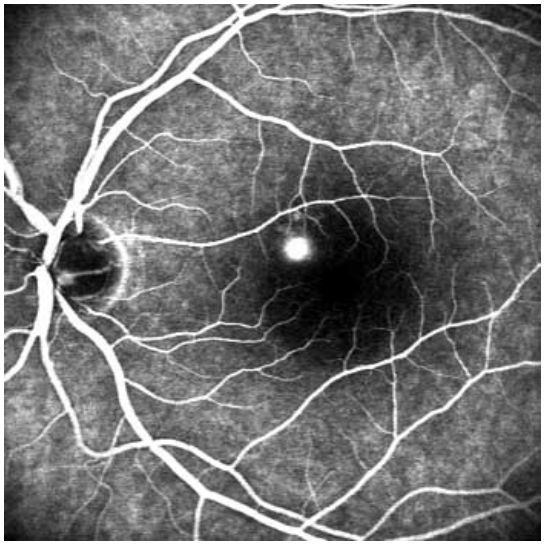

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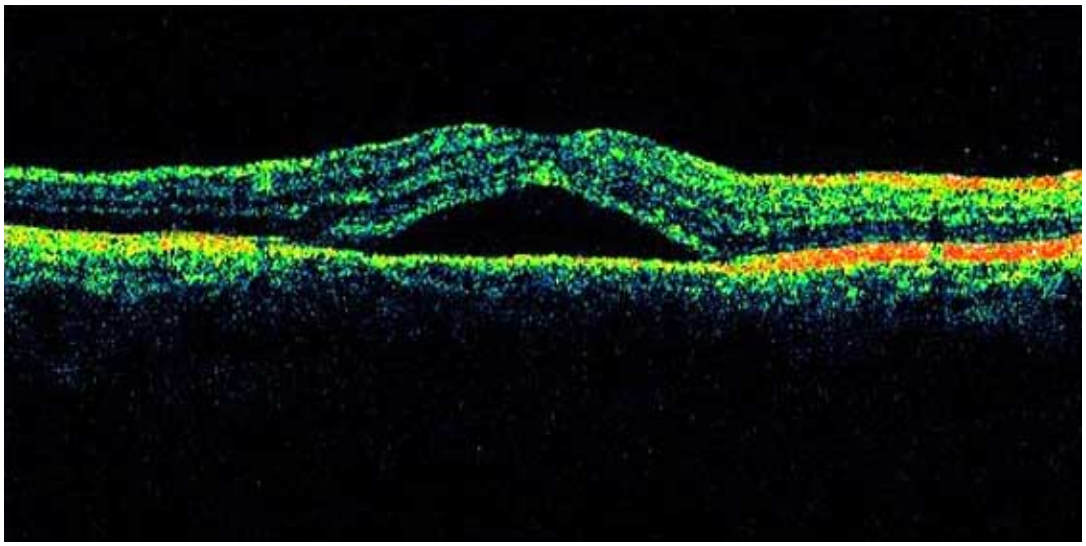
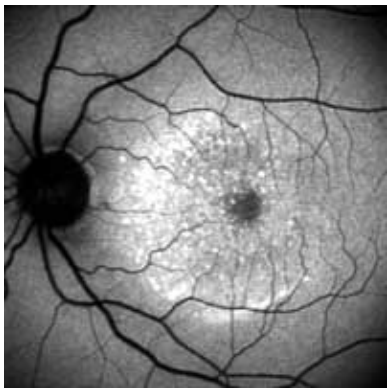


c

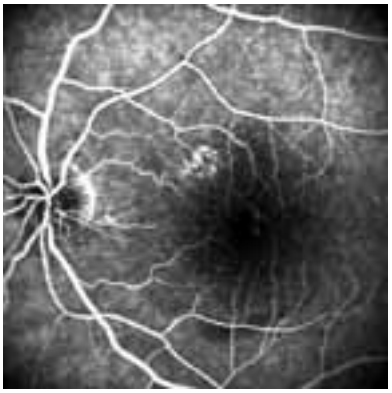
Fig. (10a, b,c). AF image, corresponding angiography and OCT in acute CSR. Significantly and well demarcated decreased AF is visible within the whole area of neurosensory detachment, which is confirmed by OCT. AF seems to be decreased due to a blocking effect of the subretinal fluid (Image from Ref. 29).

neurosensory tissue and the complete functional loss in the area of laser lesions a secondary RPE atrophy results, which needs between 6 and 12 months of time as shown in this study [25]. Thus AF can be used as a long-term monitoring tool of RPE changes after laser treatment and presumably other pathologic conditions. The enlargement of the laser atrophy zone, which was observed within the first 2-4 years potentially demonstrates the risk of visual loss after central laser photocoagulation even years after treatment (Fig. **13**).

As a result of the previously described study an interesting application of using AF is the non-invasive detection of selective RPE laser lesions in SRT. Several macular diseases are thought to be caused only by a reduced function of the RPE cells. Therefore a method for the selective destruction of the RPE cells without causing adverse effects to choroid and neuroretina, especially to the photoreceptors, seems to be an appropriate treatment [58]. The selective effect on RPE cells, which absorb about 50% of the incident light due to their high melanosome content [31] has been demonstrated using 5 µs argon laser pulses at 514nm with a repetition rate of 500 Hz [58]. By irradiating the fundus with a train of µs laser pulses it was possible to achieve high peak temperatures around the melanosomes. This led to a destruction of the RPE, but only a low sublethal



11a



11b

Fig. (11). AF image and corresponding angiography in healed CSR. No active leakage is present but only fluorescein staining in the area of former RPE defect. Increased AF is present within the former neurosensory elevation, presumably explainable due to higher metabolism of RPE cells from the protein enriched subretinal fluid (Image from Ref. 29).

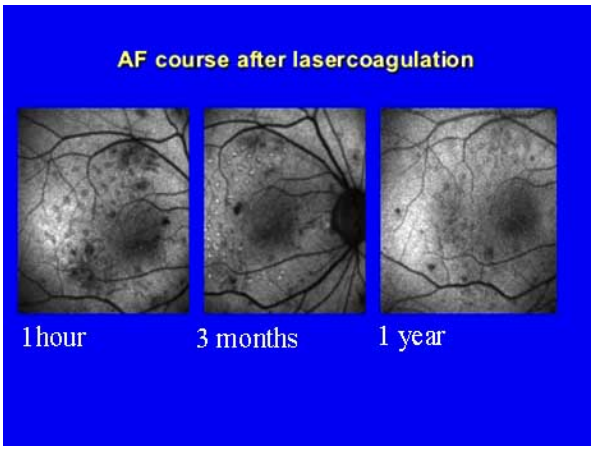


Fig. (12). Sequence of AF images showing specific patterns in conventionally applied laser lesions. After treatment lesions showed decreased AF presumably due to RPE damage and blocking of subretinal fluid. After days to months RPE proliferation to cover the defect takes place leading to significantly increased AF. Towards one year after laser treatment AF fade away due to presumed atrophy of RPE at the lasered site (Images from Ref. 25).

temperature increase in adjacent tissue structures [57]. This selective destruction of the RPE cells sparing the photoreceptors without causing laser scotoma has been proven by histologic examinations at different times after treatment [55, 57]. The first clinical trial using a Nd:YLF laser system with a pulse duration of 1, 7µs (100 pulses, 100 and 500 Hz) also proved the concept of selective RPE destruction and demonstrated the clinical potential of this technique [55, 56]. However, one of the problems concerning selective RPE laser destruction is the inability to visualize the laser lesions. Therefore it is necessary to perform fluorescein angiography after treatment to confirm the laser success and to make sure that sufficient energy was used. Since dosimetry of such laser lesions is not known, test lesions with various energy and numbers of pulses in non-significant areas of the macula – usually at the lower vessel arcade - have to be applied to elucidate the energy levels required for treatment. If the RPE is damaged, or the tight junctions of the RPE barrier are broken, fluorescein from angiography can pool from the choriocapillaris into the subretinal space. Thus fluorescein angiography has been used to detect a break of the RPE barrier. However, fluorescein angiography is an invasive method and has as already described a potential risk for allergic reactions because of the intravenous injection of the fluorescein dye. The concept of AF based detection could be proven in a pilot study [21]. For this fluorescein angiography showed leakage from the irradiated areas for about one week after treatment. None of the laser lesions was ophthalmoscopically visible during treatment. Identification of the lesions was possible by AF imaging showing an intensity decay in the irradiated area in 22 out of 26 patients, predominantly in patients with CSR and confluent soft drusen regarding to AMD. Lesions could be identified 10 minutes after treatment as spots with markedly decreased AF correlating with the area of leakage in fluorescein angiography (Fig. **14**). This was correlating with the results of conventional laser photocoagulation and proves the successful damage to the RPE. During follow up the laser spots revealed significantly increased AF which was also consisting with the behavior of conventionally applied laser coagulations and gives evidence for proliferation of RPE cells [21; Fig. **15**). Interestingly, no atrophy of RPE cells was found in several eyes after SRT in contrast to the conventional setting, which hints for the desired non-destruction of photoreceptors. Thus, imaging of non-visible selective RPE laser effects can be successfully achieved by AF measurements. Therefore, AF may also replace invasive fluorescein-angiography in many cases to verify therapeutic laser success postoperatively.

a **b**

Fig. (13a, b). Laser lesions aging several years seem to enlarge (a) and get cornered (b). Due to this behavior central photocoagulation might lead to visual loss even after years (Image adapted from Ref.25).

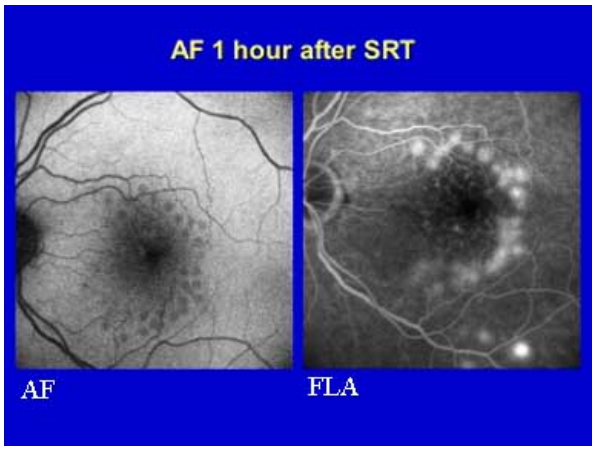


Fig. (14). AF image and corresponding fluorescein angiography 10 minutes after SRT for high-risk drusen in AMD. Grid-pattern laser lesions could clearly be identified due to significantly decreased AF corresponding with fluorescein leakage from the RPE defect (Image adapted from Ref.21).

The AF decay found postoperatively was supposed to be associated with RPE damage [21, 25]. Thus, it was questionable, if an AF decay could also be measured online directly during selective RPE laser treatment and whether this decay also contributes to the RPE damage or has a different origin. Since irradiation in conventional laser photocoagulation as well as in SRT is obtained with a laser at a green wavelength [within the excitation spectrum of AF [7, 9], it might be possible to both excite AF and to measure its intensity online during the laser treatment. Thus, possible changes in AF during irradiation might be recorded. For this a slit-lamp-adapted device for online measurement of AF during selective RPE laser irradiation was adjusted [28; Fig. **16**). In in-vitro experiments on porcine RPE sheets it was shown that AF intensity decreased as a function of the number of applied pulses. Initially, it was supposed to achieve this AF decay due to the damage of the RPE cells from the laser treatment. However, it was found that irradiation at energy levels lower than the RPE damage threshold also revealed a characteristic AF decay, which did not differ significantly from the AF decay in laser lesions with damaged RPE [28]. Thus, if the laser-induced AF decay is not related to RPE damage, another kind of interaction must be responsible for it. As derived from in-vitro experiments with A2-E, the main fluorophore of lipofuscine, the AF decay seems to be induced by a temperature increase. A2-E revealed completely reversible linear fluorescence decay under temperature increase of about 1% per 1°C [27; Fig. **17**). Thus, if those results are transferable to tissue conditions in RPE sheets and in human settings, it may be speculated that the strong AF decay is derived from the temperature increase in the irradiated tissue rather than to RPE damage. In this case, the AF decay would be reversible and completely related to temperature, independently of RPE damage. AF measurements during patient treatments, which were performed using two different parameters (100 pulses at 500Hz repetition rate and 30 pulses at 100Hz repetition rate), were expected to lead to a different increase in tissue temperature during treatment. Using the 500Hz parameter set a clear AF decay could be observed in all irradiated areas (Fig. **18**), whereas using the 100Hz repetition rate leading to a lower energy deposit in the tissue, AF changes were smaller and inconsistent. Due to these differences and derived from the A2-E results it was concluded that the AF decay in humans is also mainly due to changes in the tissue temperature during treatment. Thus AF changes seem unable to reflect RPE damage in SRT, as initially expected from the cSLO findings [21]; however it might be used as a non-

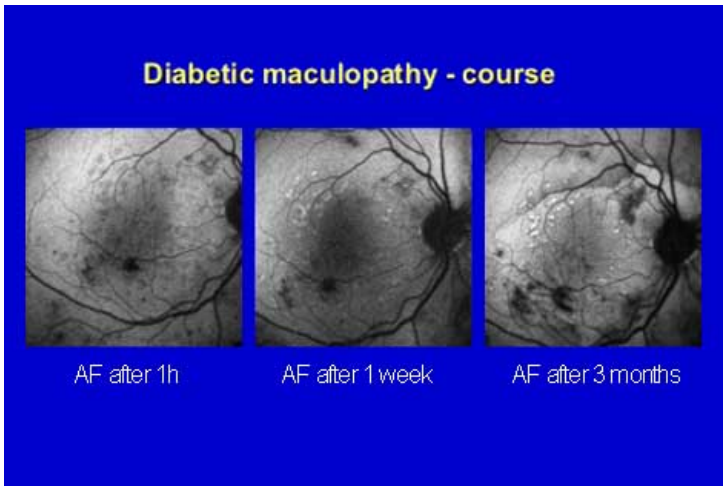


Fig. (15). Sequence of AF images after SRT. As found for conventional treatment lesions showed decreased AF presumably due to RPE damage and blocking of subretinal fluid. Consequently RPE proliferation occur leading to significantly increased AF. No atrophy of RPE derived from the AF findings was seen up to three years after treatment demonstrating selectivity of SRT.

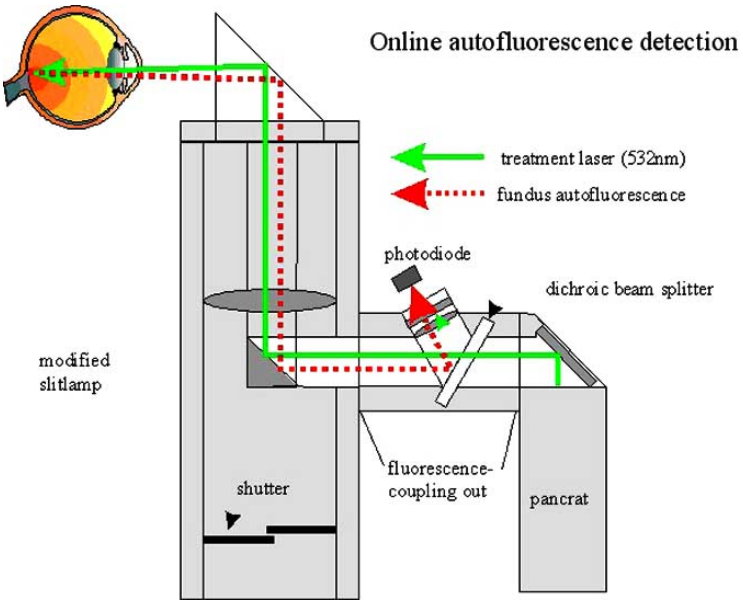


Fig. (16). Setup for online detection of autofluorescence during selective RPE laser treatment (SRT). Using a green treatment laser fundus autofluorescence is excited during irradiation, separated in the slitlamp setup by a dichroic beamsplitter and finally recorded by a photodiode (Image adapted from Ref.28).

invasive tissue temperature detection device in other laser applications as photodynamic therapy or transpupillary thermotherapy.

As described by Brinkmann *et al*., the damage mechanism in SRT is more a thermo-mechanical one than a purely thermal one as in conventional laser treatment due to the short-duration laser pulses in the microsecond-regime [5]. Thus, microbubble formation around the melanosomes inside the RPE cell occurs during treatment, probably leading to disruption of the cell; this is in contrast to thermal denaturation in conventional laser photocoagulation [5, 59]. An abrupt AF decay due to a thermal destruction of fluorophores, or due to an explosive movement of fluorophores aside - would have been expected to detect RPE damage by online AF measurements. However, those characteristics were not found, which underline the proposed tissue temperature – AF interactions. But it might be possible that an AF online based detection of SRT laser lesions can be realized if a different algorithm with e.g. time-resolved AF measurements is used. The experiments showed that it is possible to measure online AF during laser treatment and that it was also possible to detect a change of AF intensity due to the laser impact in SRT.

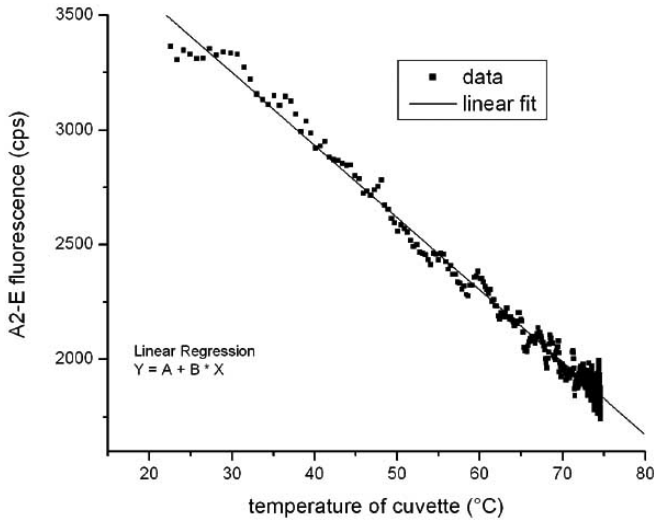


Fig. (17). Course of fluorescence intensity (cps, counts per second) of A2-E in DMSO (1µM) over temperature. A linear correlation was found between decay of fluorescence intensity and temperature increase, which was reversible (Image adapted from Ref.27).

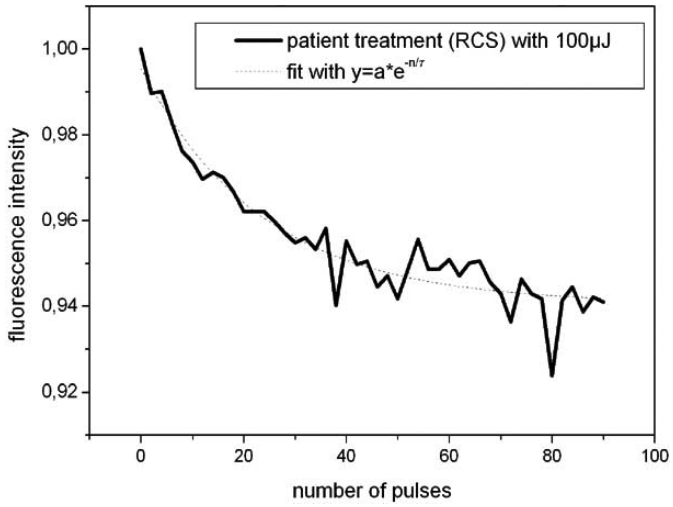


Fig. (18). Recorded online autofluorescence during a train of 100 pulses of 1.7µs pulse duration (repetition rate 500Hz). A clear intensity decay of autofluorescence was observed over the number of pulses(Image adapted from Ref.28).

Another remarkable possibility of AF is the chance to use it as a tool for determining the macular pigment density as evaluated within the last two years. Since it is known that the macular pigments as lutein and zeaxanthin function as a kind of internal sunglass preventing blue light damage to the RPE cells, it is of particular interest to define the macular pigment density non-invasively. It is supposed that a higher density leads to better prevention of AMD. Thus nowadays large efforts were undertaken to use special supplement dietary with lutein to achieve an increase of the macular pigment density. As already examined in some studies [10, 53, 82, 83] the specific macular pigment distribution profiles could be determined with AF measurements using modified scanning laser ophthalmoscopes. For this at least two different excitation wavelengths as e.g. 488nm and 514nm are necessary to achieve different AF values. Since the absorption of macular pigment at 488 nm wavelength is high and at 514 nm wavelength close to zero, macular pigment density could be determined by comparing foveal and parafoveal fluorescence at 488 nm and 514 nm [83]. As shown in different studies AF-derived density could be very well obtained using AF and it correlates with those of psychophysically obtained values [53], thus AF was regarded as a fast non-invasive method for quantitatively assessing macular pigment density *in vivo*. Differences in pigment densities could also already be evaluated between healthy subjects and patients suffering from AMD [82]. It might be interesting to see, if future studies could underline the proposed correlation of macular pigment density and onset of AMD and whether specific dietary influences the amount of macular pigments.

Finally time resolved AF measurements are an interesting new issue in imaging AF and are currently used in an experimental setup by the German study group of Schweitzer [61, 62], who determines AF of different fluorophores than lipofuscin. These coenzymes usually depend on oxygen concentration. Detection of alterations in oxygen concentrations are up to 3 orders of magnitude more sensitive by AF of coenzymes than by measurement of the natural oxygen saturation. The short fluorescence lifetime of these fluorophores being in the nanosecond-regime could be evaluated after pulse excitation [61]. Applying a laser scanner ophthalmoscope and a mode-locked argon ion laser as well as time-correlated single photon counting, lifetime images of the living fundus were obtained [61]. Those measurements might offer the possibility to evaluate cell metabolism on cellular basis and thus to detect early functional malfunctions before morphologic alterations take place [61]. Future results might therefore provide excellent prospects to determine cell metabolisms non-invasively.

In summary, AF imaging is a new interesting tool to provide information on intrinsic fluorescence mainly derived from the RPE cell layer. AF is mainly derived from the intracellular lipofuscin accumulating with age and due to alterations in different diseases. However, also other fluorophores than lipofuscin might contribute to AF. With introduction of laser scanning ophthalmoscopes evaluation of fundus autofluorescence became possible. Examination is non-invasive, cheap and fast. Proper interpretation of the images might be difficult due to previously unknown patterns and need to be learned. As shown in several examples AF adds relevant information for differential diagnosis in a variety of macular diseases. Moreover, it could be used as a monitoring tool after surgery and laser treatment. Further developments as AF based online and postoperative monitoring might contribute as adjunctive tools for new treatment modalities as SRT or tissue temperature measurements. An interesting issue is the determination of macular pigment density. AF detection of oxygen depending intracellular metabolisms might be used for prophylactic purposes in several macular diseases in future.

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