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Autofluorescence methods in ophthalmology

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Abstract. An overview of the fluorophores of the eye, the methods used to measure ocular fluorescence, and the existing or ready-to-market instrumentation for the early diagnosis and monitoring of ophthalmic pathologies is presented. Emphasis is given to the impact that a more detailed knowledge of the fluorophores of the eye, their age and pathology relationship, and the considerable technology-driven progress in optical devices and components has had on the development of new compact yet powerful instruments for population screening and for patient follow-ups, using fluorometry alone or in combination with other optical techniques such as light scattering. © *2004 Society of Photo-Optical Instrumentation Engineers.* [DOI: 10.1117/1.1628241]

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1 Introduction

Ocular pathologies such as diabetic retinopathy, glaucoma, and cataract represent the most frequent cause of blindness in the Western world. This is more and more evident in a society characterized by a constant increase in the population's average age, since most of the these pathologies are age related. Therefore, a considerable effort has to be spent in developing means to prevent the onset of sight-impairing pathologies, and to monitor their evolution in a qualitative or even quantitative way, even at a very early stage. The techniques and instruments used should then be accompanied by sophisticated, possibly noninvasive, surgical tools aimed at the treatment of ocular disturbances with minimal burden to the patient.

An increasingly outstanding role has been played in the past few years by instruments and techniques able to provide diagnostic aids to the surgeon, to clearly define degrees of severity of the disease, to identify high-risk populations, and to assess the validity of therapy through the control of drug pharmacokinetics.

Most ocular media and tissues exhibit fluorescence emission upon excitation by suitable wavelengths of light. A large variety of these ocular fluorophores exhibit marked changes in their fluorescence properties with respect to age and pathology. Thus, the biological and functional properties of many ocular media and tissues and their modifications are indicators of aging or disease and can be used as diagnostic tools.

In addition to naturally existing ocular fluorophores, a number of other fluorescing compounds may be present in the eye. Their presence can be either intentional or nonintentional. The former case includes all fluorescing drugs that are delivered to the eye for specific diagnostic purposes (e.g., as a contrast marker in fluoroangiography and fluorophotometry). The latter case includes all fluorescing compounds that are present in the eye as a consequence of diagnostic or therapeutic purposes outside the eye, and that can accumulate in the eye after crossing the ocular barriers. A third, less-studied category of fluorophores is that of vitreous substitutes. Vitreous substitutes are substances (in general, polymers or solutions) that are inserted into the eye for vitreal surgery. The fluorescence properties of these compounds are a secondary but important subject of investigation in relation to ocular fluorometry.

For both types of fluorophores, endogenous and exogenous, ocular fluorometry as a diagnostic technique is in general facilitated by the light transmission properties that the eye, as a vision organ, exhibits at most of the wavelengths of interest for fluorophore excitation and fluorescence detection (with the exception of the UV range). However, the ease of penetration of light through the ocular tissues raises strict requirements of selectivity for fluorophore discrimination.

The evolution of technology in optics, electronics, and optoelectronics has produced a tremendous amount of new light sources, detectors, modulators, and electronic circuits for signal analysis and elaboration. These can be viewed as the building blocks of a new generation of easy-to-use, powerful, and yet fairly compact and inexpensive instruments for the measurement of ocular fluorescence both *in vitro* and *in vivo*. Together with the ''traditional'' systems for ocular fluorometry, such as the well-known Fluorotron Master, new exciting clinical instruments have been put on the market, or are at an advanced state of development and engineering, almost ready for commercialization. In addition, when fluorometry is not itself sufficient for gathering adequate diagnostic information, it can be efficiently combined with other optical measurement techniques (static or dynamic light scattering, or Raman spectroscopy, are typical examples), thus providing an integrated multisensor approach. The Laboratory of Optoelectronics in Brescia, and the new Laboratory of Optoelectronics in Modena, are active in the development, characterization, and preclinical testing of systems for ophthalmic diagnosis using fluorometry, light scattering, and interferometry. This work is done in close collaboration with important laboratories at a national and international level.

This paper gives an overview of the state of the art of methods and instruments for fluorescent measurements in the eye, alone or with multisensor approaches. Section 2 is devoted to an overview of natural fluorophores, discussing their significance in relation to their application in ophthalmic di-

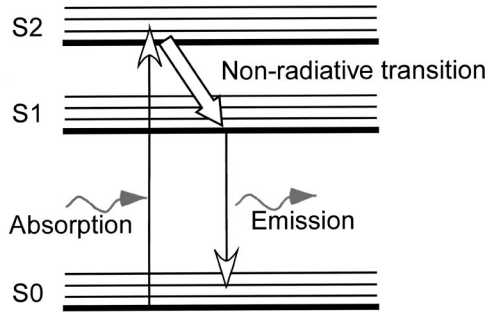

Fig. 1 A Jablonski diagram illustrates the basic mechanisms of fluorescence. Excited electrons remain in the excited state (S_1 and S_2) on a time scale of nanoseconds, then return to the ground state (S_0). When photons are emitted, their wavelengths are longer (lower frequency) and consequently they are less energetic than the photons responsible for the excitation, owing to energy loss (nonradiative transition) by the molecule prior to emission.

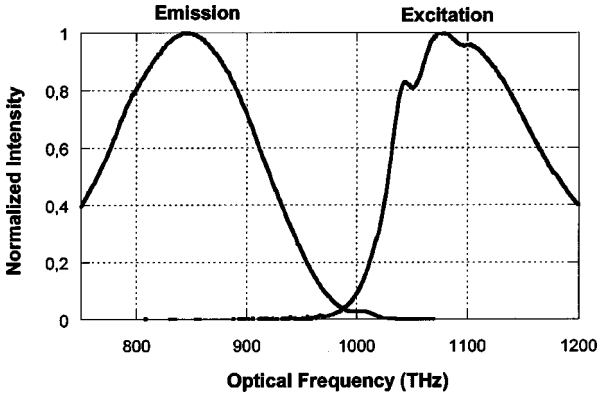

Fig. 2 Extinction coefficient and fluorescence emission spectrum of tryptophan dissolved in water, 0.1 M phosphate buffer, pH 7 using an excitation wavelength of 270 nm. Note that the extinction and the emission spectra are mirrored.

agnosis using fluorometry. Section 3 focuses on traditional and innovative fluorometric methods and instruments, and Sec. 4 describes the most significant diagnostic methods that use fluorescence measurements in the various eye compartments.

2 Natural Fluorescence of Ocular Tissues

2.1 *Basic Mechanisms*

Fluorescence is luminescence characterized by an emission of light on a short time scale after excitation. Light is absorbed by the fluorophores in femtoseconds, causing electrons to become excited to a higher electronic state. The electrons remain in the excited state for nanoseconds, then return to the ground state. Energy is emitted as the electrons return to their ground state. The whole process is frequently illustrated using the Jablonski diagram[1] shown in Fig. 1. For the most relevant molecules of interest, the lowest energy state, the ground state, is a singlet. This is normally represented as S_0, and the various excited states of the same multiplicity are shown as S_1, S_2, S_3, etc. Each state includes various vibrational levels (four in Fig. 1). As noted by Vavilov[2] and Kasha,[3] the fluorescence of ocular molecules normally involves a transition from the lowest vibrational level of the lowest excited electronic state. When photons are emitted, their wavelengths are longer (lower frequency) and consequently are less energetic than the photons responsible for the excitation; this is due to energy loss by the molecule prior to emission.[4] This difference between excitation and emission maxima is termed the Stokes shift.

The Stokes shift represents the mean energy lost while the molecule was in the excited state. The Stokes shift is important for many reasons, but from a practical point of view, it allows the emitted fluorescent photons to be easily distinguished from the excitation photons, leading to the possibility of very low backgrounds in fluorescence studies. A second important result is that the vibrational spacings in the ground (S_0) and lowest excited (S_1) states are often very similar; therefore a mirror image is often observed between the excitation and emission spectra when they are plotted as functions of the optical frequency. As an example, we show in Fig. 2 the excitation and emission spectra of one of the most famous natural ocular fluorophores: the tryptophan.[5]

An important parameter in fluorometry is the quantum yield of fluorescence, defined as

$$\Phi = \frac{N_e}{N_f}, \tag{1}$$

where N_f is the number of absorbed photons and N_e is the number of photons emitted as fluorescence. The yield of fluorescence depends upon the dynamics of the decay of the excited state caused by other competing reactions.

A spectral shift toward the red makes it possible to excite a fluorophore over a narrow spectral band and detect a much weaker fluorescence by excluding the reflected and scattered excitation using a suitable narrowband filter. The limits of detection of a fluorescence signal depend mainly on the properties of the fluorophores. A detectability to parts per billion or even parts per trillion is common for most analytes. This extraordinary sensitivity allows the reliable detection of very low concentrations of fluorescent molecules.

Fluorescence measurements can be highly specific since, even if different compounds may absorb and emit light, it is rare that their emitted light spectra overlap completely with the spectrum of the light emitted by the target fluorophore. Moreover, fluorescence intensity is linear with fluorophore concentration over a very broad range. Thus, fluorometry can be used over three to six decades of concentration according to instrumentation dynamics.

2.2 *Overview of the Ocular Endogenous Fluorophores*

Most ocular media emit natural fluorescence upon suitable excitation. The accumulation of natural fluorescent proteins in ocular tissue can result from exposure to ambient light[6,7] or to ocular pathologies.[8] As stated earlier, in addition to naturally existing ocular fluorophores, a number of other artificial fluorescing compounds may be present in the eye. They can be either intentional (e.g., as a contrast marker in fluoroangiography) or nonintentional (e.g., fluorescing compounds used for diagnostic or therapeutic purposes that, after crossing the ocular barriers, accumulate in the eye). This section focuses

Table 1 Main characteristics of the ocular endogenous fluorophores.

Ocular Tissue	Endogenous Fluorophores	Excitation and Emission Wavelengths (nm)
Cornea	Flavoproteins	$\lambda_{ex}=450$ $\lambda_{em}=550$
	Pyridine nucleotides	$\lambda_{ex}=366$ $\lambda_{em}=450$
	Glycosylated collagen	$\lambda_{ex}=370$ $\lambda_{em}=440$
Cristallin lens	Tryptophan	$\lambda_{ex}=295$ $\lambda_{em}=329$
	Nontryptophan	$\lambda_{ex}=364$ to 472 $\lambda_{em}=437$ to 523
	Hydroxy-kynurenine glucoside	$\lambda_{ex}=520$ $\lambda_{em}=550$
Vitreous body	—	—
Retinal pigment epithelium	Lipofuscin	$\lambda_{ex}=300$ $\lambda_{em}=420$
	Melanolipofuscin	$\lambda_{ex}=364$ $\lambda_{em}=540$

on the naturally fluorescing compounds called endogenous fluorophores (autofluorescent compounds). These fluorophores are visible in the cornea, lens, vitreous body, and retinal pigment epithelium (RPE). In the next sections, ocular endogenous fluorophores located among the different ocular tissues are presented. Table 1 summarizes the main characteristics of the fluorophores described.

2.2.1 *Cornea*

Corneal autofluorescence was demonstrated to originate from flavoproteins and pyridine nucleotides as a result of metabolically impaired corneal mitochondrial respiration.[9] Endogenous fluorophores in the cornea are located in the corneal epithelium and endothelium. These fluorophores are mainly flavoproteins (flavine adenine dinucleotide or FAD, and flavin mononucleotide or FMN) and pyridine nucleotides (nicotinamide dinucleotide or NAD and nicotinamide dinucleotide phosphate or NADP).[9,10]

The flavoproteins fluoresce in their oxidized form (FAD and FMN) with an excitation maximum at $\lambda_{ex}=450$ nm and an emission maximum at $\lambda_{em}=550$ nm, whereas pyridine nucleotides fluoresce in the reduced state (NADH and NADPH; $\lambda_{ex}=366$ nm and $\lambda_{em}=450$ nm).

Fluorescence of the cornea can also originate from glycosylated collagen via the Maillard reaction.[11] The excitation maximum of collagen-linked fluorescence is 370 nm, with an emission maximum at 440 nm.[9]

2.2.2 *Cristallin lens*

The α, β, and γ crystallins in the lens exhibit tryptophan fluorescence ($\lambda_{ex}=295$ nm and $\lambda_{em}=329$ nm). The distribution of tryptophan fluorescence is fairly uniform throughout the lens cross-section,[12] mirroring the distribution of proteins.[13] Nontryptophan fluorophores are not present in the newborn and fetal lenses.[14]

Lens aging favors protein bonds and thus there is an increased amount of water-insoluble protein fractions (up to 40% of the overall amount of lens proteins).[15] The development and accumulation of nonsoluble protein fractions in the lens have been demonstrated to be the result of cross-linking of water-soluble proteins through photo-oxidation processes. Photo-oxidation leads to disulfide or other bonds that are supposed to be initiated by the combined action of long-wavelength UV light and endogenous photosensitizers such as kynurenine and bityrosine.[14,16,17] Consequently, nontryptophan fluorescence develops and increases with age.[14,18,19] Besides photochemical processes, metabolic processes may contribute in part to the buildup of nontryptophan fluorescence.[16,19,20] Excitation and emission spectra of nontryptophan fluorescence exhibit peaks at longer wavelengths than tryptophan; typically $\lambda_{ex}=364$ to 472 nm and $\lambda_{em}=437$ to 523 nm. Nontryptophan fluorescence is markedly nonuniform throughout the lens, decreasing from the outermost layers to the nucleus with axial symmetry.[12,21]

Fluorescence of the lens can also originate from the glucoside of hydroxykynurenine (3-HGK), which is a nonprotein-bound, nontryptophan fluorophore present even in young eyes.[16] The excitation maximum of the glucoside of hydroxykynurenine fluorescence is 520 nm, with an emission maximum at 550 nm.[22]

2.2.3 *Vitreous body*

The literature does not report on the fluorescence properties of vitreous, which is currently considered as nonfluorescent in the visible. However, there is evidence of fluorescence exhibited by vitreous, especially in older eyes, as well as of age-related modifications of these fluorescence properties. To our knowledge, no attempt has been made to identify the nature of the fluorophores of the vitreous.

2.2.4 *Retinal pigment epithelium*

Fluorescence of the retinal pigment epithelium is mainly related to lipofuscin. Intact human lipofuscin granules exhibit a broadband excitation spectrum from 300 to about 420 nm, and an emission spectrum that peaks in the yellow-orange region of the spectrum. Both excitation and emission spectra are age related.[23,24]

Melanin is the other main chromophore of the RPE; it is generally considered as nonfluorescent. However, studies on melanin granules *in vitro* showed age-related fluorescence properties, probably due to some combination with lipofuscin. The excitation maximum of melano-lipofuscin fluorescence is 364 nm, with an emission maximum at 540 nm.[22]

3 Instrumentation for Ocular Autofluorescence Measurements

3.1 *State of the Art*

Ophthalmic diagnostic methods are based on the well-known continuous wave (cw) technique for analytical *in vitro* tests: single-point measurements and image measurements. In the following paragraphs, some of the best-established instrumen-

Table 2 Main characteristics of the instruments for ocular autofluorescence measurements.

Diagnostic Method	Laboratory or Commercial Instrument	Excitation Source	Emission Detector	Ocular Tissue Investigated
Axial-scanning fluorometry (ASF)	Fluorotron Master (Ocumetrics, Inc.)	Halogen lamp	Single-photon counting photomultiplier tube	Axial scan of the ocular axis (from retina to cornea)
Imaging ocular fluorometry (IOF)	Modified slit lamp Scheimpflug camera (Topcon SL45)	Halogen lamp Halogen lamp	Analog photodetector or camera CCD cameras	Cornea and cristallin lens
Confocal imaging fluorometry (CIF)	Laser scanning ophthalmoscope (Heidelberg Engineering)	Lasers	Photomultiplier tube or photodiode	Retinal pigment epithelium
Optical multichannel analyzer (OMA)	Modified slit lamp	Halogen lamp	Multichannel photodetector	Cristallin lens
Corneal fluorometry (CF)	Portable instrument developed by the Laboratory of Optoelectronics (University of Brescia)	Light-emitting diodes (LEDs)	Analog photomultiplier tube	Cornea
Autofluorescence combined with dynamic light scattering (AF+DLS)	Integrated instrument developed by the Laboratory of Optoelectronics (University of Brescia)	Halogen lamp and laser	Single-photon-counting photomultiplier tube and single-photon-counting avalanche photodiode	Axial scan of the ocular axis (from retina to cornea)

tation performing *in vivo* autofluorescence measurements is presented. Table 2 summarizes the main characteristics of these instruments.

3.1.1 *Axial-scan fluorometry*

Axial-scanning fluorometric (ASF) techniques detect autofluorescence intensity as a function of the scanning coordinate to quantify alterations in ocular tissues. While various systems have been suggested, probably the most popular is that suggested by Zeimer et al.[25] and commercially available as the Fluorotron Master (FM; Ocumetrics Inc., California). This system was originally designed to measure fluorescein within the eye. Basically, the output data are the fluorescein concentration profile along the optical axis (from retina to cornea). As shown in previous studies,[26] the excellent sensitivity of the FM also makes it suitable for measuring autofluorescence of the ocular tissues. The system sends a focused excitation beam of blue light into the eye and then detects the resulting naturally fluorescent green light. In the standard operation mode, up to 149 sequential measuring positions are explored along the ocular axis. The measuring range is 37 mm, which is sufficient to scan the eye from the retina to the cornea, whereas the scanning spatial resolution is 0.25 mm. The excitation source consists of a tungsten light source followed by the excitation bandpass filters (440 to 480 nm). The medical operator can scan the patient's ocular axis from the retina to the cornea. The resulting natural fluorescence is collected by the same lens system and guided to the emission optical filter (530 to 630 nm). The filtered optical signal is detected by a photomultiplier tube operating in a single-photon counting mode. By means of suitable calibration tools, including plastic fluorescent samples, the instruments readings are provided directly in terms of concentration of fluorescein.

3.1.2 *Imaging ocular fluorometry*

The most popular imaging ocular fluorometry (IOF) techniques are based upon modification of standard slit-lamp photographic techniques. The slit lamp, usually a halogen lamp equipped with suitable filters, is used as the excitation source. The fluorescent emission is imaged onto the detector or the camera, which is placed at an angle with respect to the direction of incidence of the light. For obvious reasons, this method is restricted to the anterior chamber and the lens, and is commonly used to monitor and record corneal and lenticular fluorescence. For some clinical applications, the fluorescence from one point of the lens section is assumed to be directly related to the amount of light reaching that point; in this case, fluorescence is used to monitor lens transmission and its age and pathology relationship.

Another important IOF technique is based on Scheimpflug photography. The optical axis of the excitation light and the axis of the film and detector equipment are parallel to each other. A long depth-of-field objective located at an angle with respect to the axis of the excitation light is used to image the scatter or fluorescence from the lens onto the film.[27,28] The Topcon SL45 camera, modified for Scheimpflug photographs, is the current instrument available for fluorescence imaging. Microdensitometric analysis and computer analysis of the photographs,[29] or direct digital acquisition using CCD cameras,[30] are used to provide a digital representation of the images.

Scheimpflug photography is at present becoming a standard method for monitoring modifications of the lens fluorescence in relation to age and cataract formation.[31–33] This is normally correlated with visible Scheimpflug densitometry to monitor lens opacification and scattering.[34,35]

3.1.3 *Confocal imaging fluorometry*

In the confocal imaging fluorometer (CIF), the excitation light is focused in a small area and the collection optics images this area on the sensitive area of the photodetector. A complete characterization of the collection optics can be performed by replacing the detector with an appropriate light source and defining the observation beam in this way. The volume under test is defined by the overlap between the excitation and the observation beam in the focus position. To obtain fluorescence images, the illuminated area and the observation beam scan the region of interest. For confocal fluorometry, laser light is more advantageous than lamp light, since a higher spatial resolution is achievable.

Recently several confocal fluorescence measurements have been performed using laser scanning ophthalmoscopes (LSO) or near-field scanning microscopes.[36,37] These instruments usually integrate different excitation lasers, e.g., argon lasers ($\lambda = 488$ and 514 nm), helium:neon (He:Ne) lasers ($\lambda = 633$ nm), and laser diodes ($\lambda = 680$ to 900 nm). In addition, two to four confocal diaphragms, acousto-optical modulation of the laser beam, and ametropia correction are used to obtain excellent fluorescent mapping in different conditions.

3.2 *Artifacts in Autofluorescence Measurements*

In vivo ocular fluorometric techniques base their effectiveness on the ability to couple excitation light of a suitable wavelength to the target, and to detect the fluorescent emission from the target area in a sensitive and selective way.

The first instrumental artifacts observed during fluorescence measurements are related to stray light in the measuring system[38] and to ambient light. Moreover, during the aging of the excitation source, the intensity of its radiation decreases and its spectral composition may change. Fluctuations in power supply can also result in different levels of brightness and spectral variations. These may affect the measurements, since autofluorescence intensity is proportional to the intensity of the exciting radiation. Therefore great care should be taken to monitor the excitation optical power and spectrum. On the other hand, the detectors also could present different sensitivity during aging. This factor is more difficult to monitor.

Another important factor affecting fluorescence measurements is the fraction of the excitation beam back-reflected in the optical path of the emission by interfaces. Even if this light has the same wavelength as the excitation beam and thus is stopped by the emission filter, this radiation could interfere with the measurement in the case of weak fluorescence signals, e.g., corneal fluorescence. Another unwanted contribution to the fluorescence signal comes from Raman scattering. In this case, a small part of the excitation scattered light is at longer wavelengths. This light is not stopped by the emission filter, and therefore the distinction between autofluorescence and Raman scattering could be a problem.

A last important contribution affecting measurements of fluorescence intensities is related to the attenuation of the excitation light beam in the optical path by the ocular tissue under test (inner-filter effect[39]). This leads to a nonlinear relation between fluorescence intensity and fluorophore concentration and, at sufficiently high concentrations, to an apparent fluorescence quenching.

In addition to instrumental artifacts, eye movements could be critical. Autofluorescence measurements may require a long measuring time; therefore spatial resolution could be largely compromised by eye movements. Moreover, unwanted ocular tissues could contribute to the total fluorescence signal detected.

3.3 *Instrument Requirements*

Systems used for autofluorescence measurements are usually highly reliable. However, measurement uncertainty and errors should be considered when interpreting data. Sources of error can be found in deficiencies of the device and/or have their origin in eye movements.

Design efforts should be made to eliminate any transmission of reflected or scattered excitation light through the emission filter, which leads to pseudofluorescence. The instrument should use high-quality interference filters for setting the wavelengths of excited and emitted light. The overlap of their transmission bands should be minimal. Munnerlyn et al.[40] recommend increasing the separation between the excitation and barrier filters to about 30 nm at the 50% transmission points and possibly doubling both filters for maximum reduction in the overlap.

A calibration procedure is essential to correct for the alteration through aging of the optical source and the detectors. Standard calibration procedure exploits commercially available fluorescent solutions, e.g., fluorescein solutions of known concentration and constant pH. This also offers the chance to convert readings to units of concentration of fluorescein and makes it easier to compare results with those obtained by other researchers. Solid fluorescence phantoms often represent an excellent and stable calibration sample.[41]

In ocular fluorometry, the optical power of the excitation must be kept within the maximum exposure limits recommended by international standards, e.g., ANSI standards.[42] However, a reduction in this optical power diminishes the available fluorescence signal, thus increasing the minimum detectable signal or increasing the time required to make a measurement. The use of lasers improves (1) the degree of spatial resolution; (2) the sensitivity; and (3) the spectral resolution, which allows unwanted contributions to fluorescence and scattering to be eliminated. However, for diagnostic purposes, gas lasers have so far found limited commercial applications because of their relatively high cost compared with standard excitation sources (lamps). For this reason we believe the semiconductor coherent and incoherent sources to be of great importance in future instrumentation for ocular fluorometry. Recently, blue light-emitting diodes (LEDs) have been proposed for measurements of corneal autofluorescence.[41] Spot size and depth of focus of the excitation beam should be designed to achieve the best spatial resolution. This can be obtained by properly shaping the excitation beam, e.g., reducing the width of the excitation diaphragm and increasing the angle between the exciting and the emitted beam, which determines the depth of the ''focal diamond.'' [40]

The first requirement for the detection system is the front-end electronics, which should be designed to maximize the signal-to-noise ratio. Photon-counting detection techniques, in which each photon represents a single digital pulse, are used to effectively reduce noise and improve sensitivity. This

means of detection would also be very useful in modern systems, in which all of the signals are digitized and processed by a computer. Photodetectors preferably should have internal amplification of the signal. Modern solid-state technology offers high-performance detectors, such as avalanche photodiodes (APD), to replace the standard photomultiplier tube (PMT).

Computerized systems improve the quality of the diagnosis in ophthalmic practice by using the computational power and storage capacity of the new personal computers. An automated comparison of two or more fluorescence signals can be easily done by a computer. Software should be user friendly and object oriented. Moreover, the system should not burden the patient, and a suitable fixation aid should be implemented before and during the measurement so that the eye is oriented correctly with respect to the optical axis.

3.4 *Outlook for New Systems and Techniques*

3.4.1 *Optical multichannel analyzer*

A fluorescence spectrometer has been specially designed for *in vivo* measurements of lens autofluorescence. It consists of a modified slit lamp integrated with an optical multichannel analyzer (OMA).[43] Even if the first model is not recent, this spectroscopic approach is still innovative for *in vivo* investigation of interactions among the various fluorophores generated in the lens. The OMA offers high sensitivity and high spectral resolution. Several studies *in vitro* have been performed and published (e.g., Refs. 43 and 44). The system was also used to perform *in vivo* measurement in various lens conditions, as described in detail in Figs. 3(a), 3(b), and 3(c).[22]

3.4.2 *Corneal fluorometer*

A portable corneal fluorometer (CF) has been developed in our laboratories. This instrument has been designed to measure the autofluorescence of the cornea.[41] As shown in Fig. 4(a), the laboratory prototype of the system is based on a photographic camera equipped with a fluorescence excitation adapter for the tangential illumination of the corneal tissue, with appropriate excitation and barrier filters, and with a photomultiplier in the image plane of the camera for the detection of the emitted fluorescence. To minimize the contribution of the much higher fluorescence of the underlying crystalline lens, special geometry and measurement procedures have been designed.

To reduce costs, custom light-emitting diodes in combination with suitable, inexpensive color filters are used as the excitation source. Custom signal-conditioning electronics has been developed to treat the signal for subsequent conversion. A single-chip microprocessor unit has been designed to control signal acquisition, to convert the acquired signal to digital form, and to display the results. To add flexibility, the instrument can be interfaced to a personal computer. Figure 4(b) shows a typical tangential illumination of the cornea performed on a bovine enucleated eye.

This portable corneal fluorometer may be successfully used in the routine screening of populations at risk for diabetic retinopathy. The apparatus has proven to be very easy to use, automatic, flexible, sensitive, linear, and repeatable.

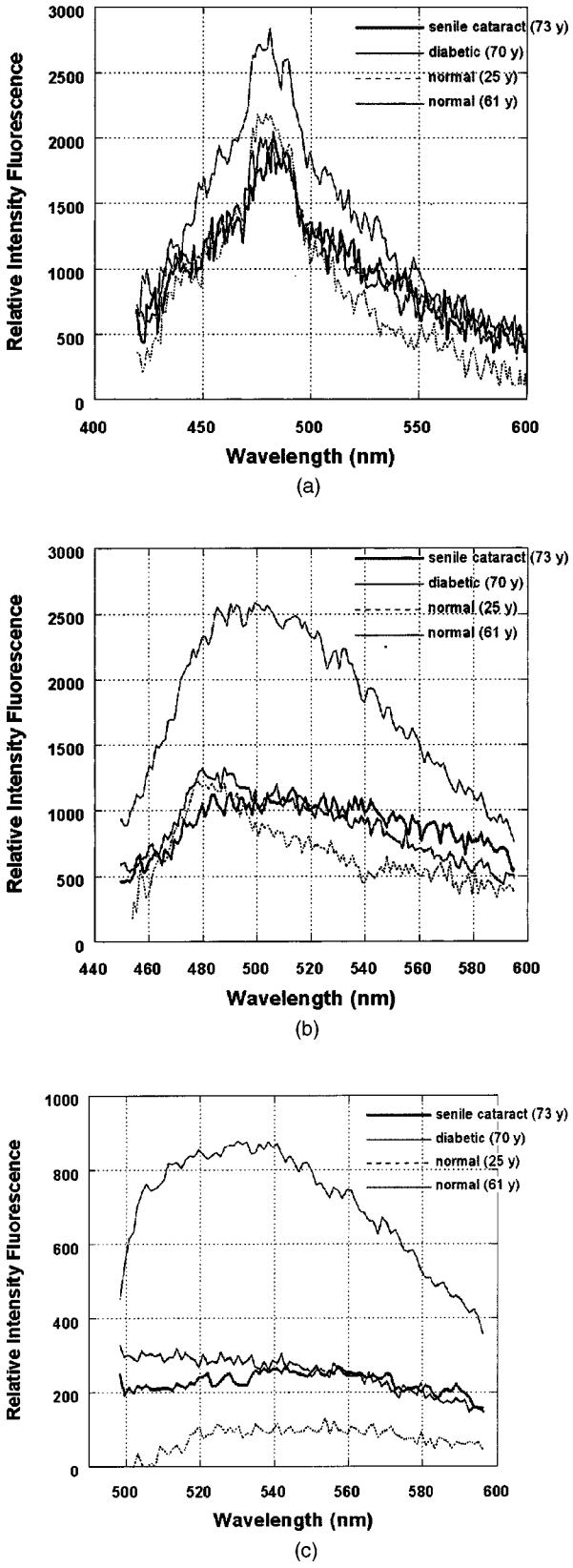

Fig. 3 (a) Comparative spectra obtained *in vivo* upon excitation at 404 nm. (b) Comparative spectra obtained *in vivo* upon excitation at 436 nm. (c) Comparative spectra obtained *in vivo* upon excitation at 485 nm. (Reproduced from Ref. 22 with permission.)

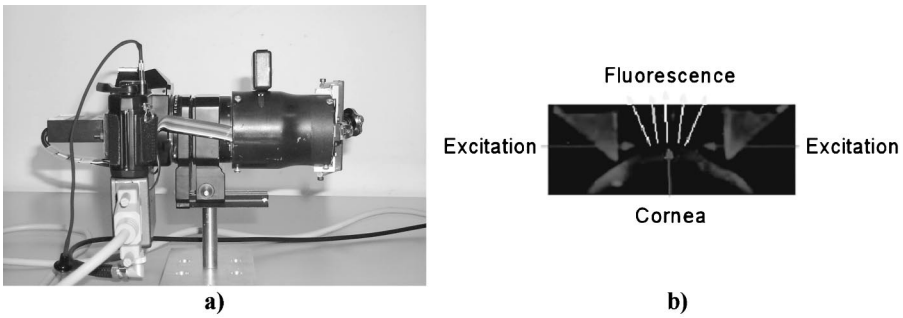

Fig. 4 (a) Portable corneal fluorometer developed in the Laboratory of Optoelectronics of the University of Brescia. (b) Typical tangential illumination of the cornea performed on a bovine enucleated eye.

3.4.3 *Axial-scan fluorometer integrated with a dynamic light-scattering analyzer*

Dynamic light scattering (DLS) is a technique for measuring the random thermal movements of suspended particles, macromolecule solutions, and other complex liquid systems.[45] Our groups developed an integrated instrument based on the FM modified to include the DLS setup.[46] The instrument simultaneously investigates the autofluorescence and DLS properties of the same ocular volume.

A block diagram of the optical setup is shown in Fig. 5(a). Optical integration between the autofluorescence and DLS system is obtained using a couple of collimators and a couple of dichroic mirrors. These mirrors, inserted between the fluorescence unit and the scanning lens SL, separate the autofluorescence and the DLS signals, offering a total reflectance to the DLS laser wavelength and a total transmittance to the autofluorescence excitation and emission beams. The DLS excitation laser source emits radiation at wavelength of 670 nm. The scattered light is detected by an avalanche photodiode. Both the excitation laser light and the scattered light are guided by single-mode optical fibers. Figure 5(b) shows the integrated instrument.

4 Overview of Autofluorescence Diagnostic Methods

As stated earlier, ocular pathologies such as diabetic retinopathy, glaucoma, and cataract can induce changes in the fluorescence properties of a large variety of ocular fluorophores. Thus the biological and functional properties of these tissues and their modifications are indicators of aging or disease and can be used as diagnostic tools. In the following paragraphs, some of the best established diagnostic methods are presented. Table 3 summarizes the main characteristics of these methods.

4.1 *Corneal Autofluorescence*

In 1990 Stolwijk[47] first proved that the weak signal, comparable to about 10 ng of fluorescein per milliliter, measured by ASF in the axial position of the cornea did not originate from excitation light scattered at the corneal surface, as previously stated,[48] but mainly originated in corneal autofluorescence.

The fluorescence arising from flavoproteins can give information about the metabolic state of the cornea. A decreased mitochondrial function, induced for instance by hypoxia, will result in a decrease in the fluorescence signal of oxidized flavoproteins.[49] An increased metabolism will result in an increased mitochondrial function, causing a higher fluorescence of the oxidized flavoproteins.[50]

As a result of aging, the amount of glycosylation collagen increases.[47] Furthermore, in diabetic patients, an increase in collagen fluorescence of the dura mater and skin was found.[51,52] The age-corrected, collagen-linked fluorescence in the skin correlated well with duration of diabetes, degree of retinopathy, arterial stiffness, and joint stiffness.

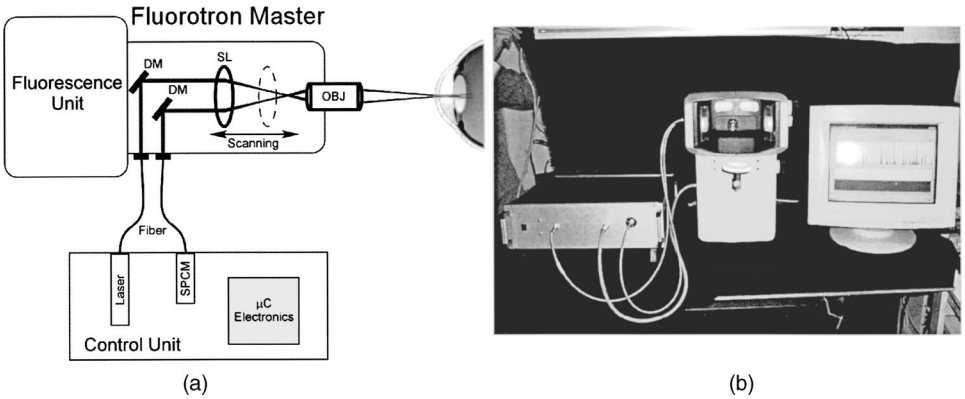

Fig. 5 (a) Block diagram of the integrated instrument based on the Fluorotron Master modified to include the dynamic light-scattering setup. DM, dichroic mirrors; SL, scanning lens; OBJ, objective; SPCM, single-photon counting module. (b) Picture of the integrated instrument. The instrument simultaneously investigates the autofluorescence and dynamic light-scattering properties of the same ocular volume.

Table 3 Main characteristics of the autofluorescence diagnostic methods.

Ocular Tissue Investigated	Pathology	Biochemical Mechanism (P, proved, S, speculative)	Instrumentation	Major References
Cornea	Diabetes	P: glycosylation of collagen	ASF	47, 53, 54
	Diabetic retinopathy	S1: elevation of blood glucose level induces alterations in lipid and protein metabolism S2: neovascularization of the iris (rubeosis iridis) may reach the cornea	ASF, CF and AF+DLS	General: 53, 54, 55 S1: 56 S2: 57
	Open-angle glaucoma	S: autonomic neuropathy causing change in corneal innervation and consequently altering corneal metabolism	ASF	63, 64, 65, 66
	Penetrating keratoplasty	S: surgical denervation	ASF	67, 68
Cristallin lens	Diabetes	P: glycosylation of collagen	ASF, IOF, and OMA	48, 78, 79
	Cataract	P: protein bonding	ASF, IOF, and OMA	73, 74, 80
Retinal pigment epithelium	Age-related maculopathy	P: accumulation of lipofuscin	CIF	89, 90
	Senile macular degeneration	P: accumulation of lipofuscin	CIF	89
	Retinitis pigmentosa	P: accumulation of lipofuscin	CIF	87
	Sjögren-Larsson syndrome	P: accumulation of lipofuscin	CIF	88

Corneal autofluorescence was found to be not dependent on age in healthy controls, non-insulin-dependent diabetes mellitus (NIDDM), insulin-dependent diabetes mellitus (IDDM), and penetrating keratoplasty (PKP) patients.[47] As shown in Fig. 6, the values for the NIDDM, IDDM, and penetrating keratoplasty patients differed significantly from those of the healthy controls (+31, +50, and +31%, respectively).[47]

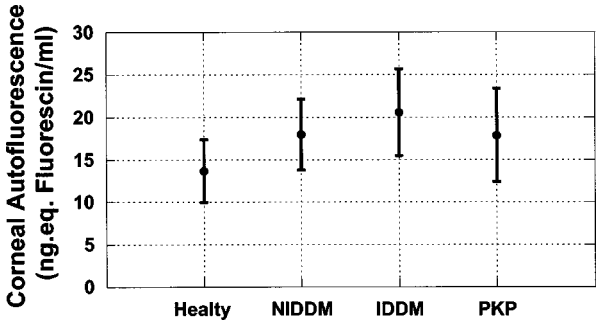

Fig. 6 Corneal autofluorescence in healthy controls, diabetics (non-insulin-dependent diabetes mellitus, NIDDM and insulin-dependent diabetes mellitus, IDDM), and patients with penetrating keratoplasty (PKP). The solid dots represent the mean values observed for each group of patients; the bars are the standard deviations.

Corneal autofluorescence was found to be strongly related to the grade of retinopathy.[53] Patients with negligible retinopathy or none (grade 1) had about the same fluorescence values as healthy controls.[54]

As shown in Fig. 7, an average increase of about 50.7% in the corneal autofluorescence between patients with negligible diabetic retinopathy (grade 1) and those with proliferative diabetic retinopathy (grade 4) was observed using a cornea fluorometer whereas, considering only IDDM patients, an average increase of about 95.8% in the corneal autofluorescence between diabetic retinopathy grade 1 and diabetic retinopathy grade 4 patients was observed.[55]

The correlation between the grade of diabetic retinopathy and corneal autofluorescence suggests, as a first explanation, that the vascular component of diabetes mellitus causing specific microangiopathy and consequently progressive retinopathy may be related to the metabolic disorder (the elevation of blood glucose level associated with alterations in lipid and protein metabolism).[56] This metabolic disorder can also affect the cornea, resulting in an increase in corneal autofluorescence. As a second explanation, neovascularization-mediating substances produced in a retina with diabetic retinopathy that induce neovascularization of the iris (rubeosis iridis) may reach the cornea as well and consequently induce changes in

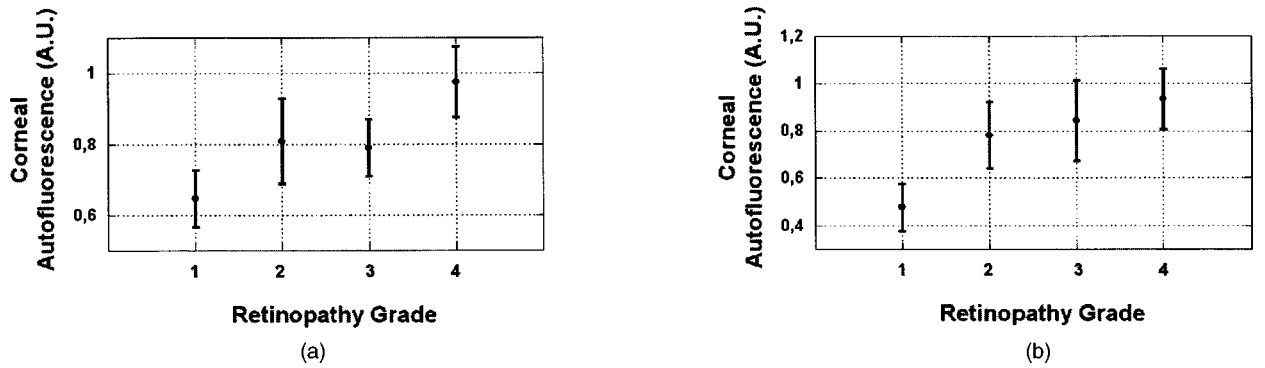

Fig. 7 Corneal fluorometer readings as a function of the retinopathy grade for the whole patient group (NIDDM and IDDM) (a) Corneal fluorometer readings as a function of the retinopathy grade for IDDM patients. (b) Solid dots represent the mean values observed for each group of patients; the bars are the standard deviations. (Reproduced from Ref. 55 with permission.)

corneal metabolism, resulting in increased values of autofluorescence.[57]

DLS combined with an axial-scan fluorometer (AF +DLS) has been used to efficiently detect early stages of diabetic retinopathy.[57] Two DLS measurements in the posterior part of the vitreous and one in the cornea were performed in each eye. The results were processed in terms of the distribution of relaxation rate. As shown in Fig. 8, a typical spectrum exhibits a sequence of peaks with different amplitudes. Two parameters were used to characterized the ocular tissues under test: (1) the abscissa Γ [max, measuring position] of the peak at the maximum amplitude and (2) the center of gravity of the relaxation rate distribution, abscissa Γ (g, measuring position). Metabolic changes that were due to diabetic retinopathy were observed using both DLS in the corneal tissue and corneal autofluorescence. High sensitivity and specificity could be obtained from corneal autofluorescence and Γ [g, cornea], by fixing a proper threshold to discriminate negligible (grade 1) from background diabetic retinopathy (grade 2). These two parameters are not suitable for assigning a diabetic retinopathy grade, since (pre-) proliferative diabetic retinopathy (grade 3) could easily be confused with negligible diabetic retinopathy (grade 1).

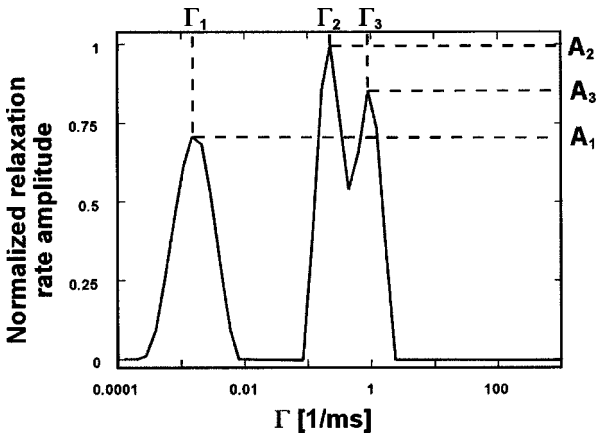

Fig. 8 Typical spectrum of the relaxation rate obtained performing dynamic light-scattering measurements in the vitreous body. (Reproduced from Ref. 57 with permission.)

The increase in the corneal autofluorescence between patients with negligible diabetic retinopathy (grade 1) and those with background diabetic retinopathy (grade 2) was associated with a marked decrease in the mean value of Γ [g, cornea]. This could be due to a metabolic disorder that results in an increase in the mean radius of the scattering molecules. However, it could also be related to a decrease in the molecular mobility, due for example, to an increase in the local viscosity or to interactions among scattering centers. Note that the decrease in the corneal autofluorescence between patients with background diabetic retinopathy (grade 2) and those with (pre-) proliferative diabetic retinopathy (grade 3) is associated with an increase in Γ [g, cornea] in the cornea. As shown in Fig. 9(a), the high correlation between corneal autofluorescence mean values and Γ [g, cornea] demonstrates that changes in autofluorescence are induced by a structural change in the corneal tissue.[57]

Glycosylation of the hyaluronate molecules as well as the presence of pathological plasma proteins, which may occur with a partial breakdown of the blood-retinal barrier (BRB) during the course of diabetes mellitus, alters the tertiary and quaternary structure of the vitreous molecules as well as their diffusivity. Such changes are known from other tissues.[58] In the vitreous they can be detected by DLS at a time when there is no visible alteration and no onset of diabetic retinopathy. Because the breakdown of BRB causes significant changes in protein concentration in the vitreous first and then alterations of the corneal tissue, DLS in the vitreous should be more effective for an early detection of diabetic retinopathy.

It may be assumed that alterations that are due to diabetic retinopathy show up in the vitreous before the cornea because of the close spatial location of the vitreous and the retina. The increase of the Γ [max, vitreous] in patients with negligible diabetic retinopathy (grade 1) and those with background diabetic retinopathy (grade 2) could be due to alteration of the tertiary and quaternary structure of the vitreous molecules as well as their diffusivity. As shown in Fig. 9(b), even the mean value of Γ [max, vitreous] significantly correlates with corneal autofluorescence, confirming that the two phenomena are strictly connected.[57]

Patients affected by open-angle glaucoma often have to use intraocular pressure (IOP)-lowering medication for several years. As Van Best has correctly stated, timolol is one of the

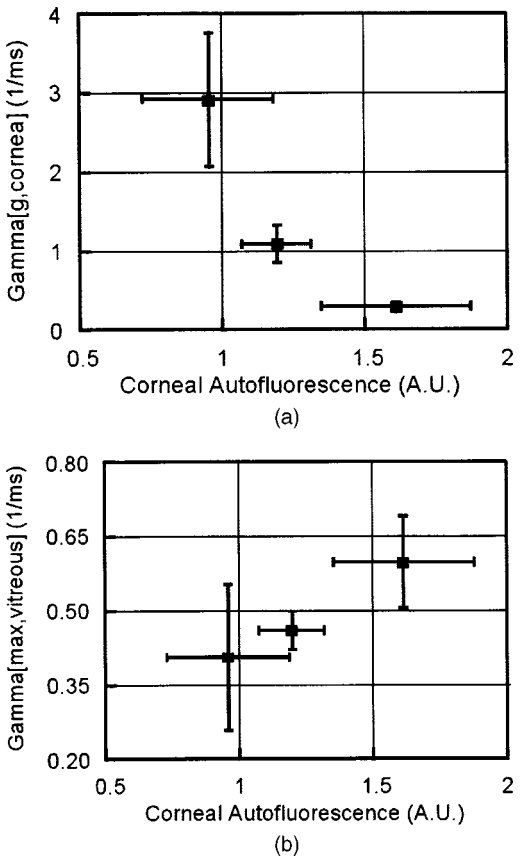

Fig. 9 (a) Γ[g, cornea] versus corneal autofluorescence in each diabetic retinopathy grade. (b) Γ[max, vitreous] versus corneal autofluorescence in each diabetic retinopathy grade. The solid squares and bars represent the mean value and the standard error of the mean in the three diabetic retinopathy groups considered in the study performed by Rovati et al. (Reproduced from Ref. 57 with permission.)

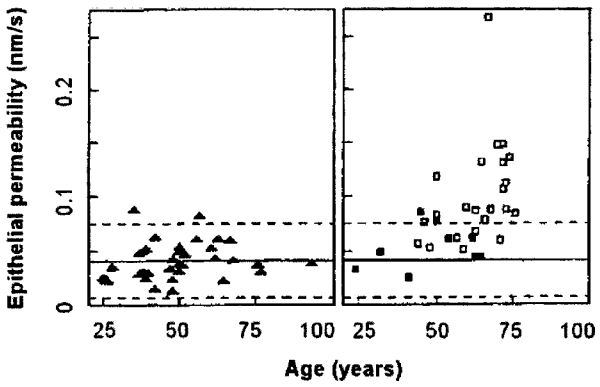

Fig. 10 Corneal epithelial permeability evaluated by autofluorescence measurements on open-angle glaucoma and ocular hypertension patients as a function of age. Solid and open squares represent patients not using and patients using timolol medication, respectively. The straight and broken lines represent the mean value and the 95% probability range of healthy control values, shown as solid triangles. (Reproduced from Ref. 22 with permission.)

most frequently prescribed eyedrops in the treatment of IOP. It can cause corneal epithelial cell damage in healthy subjects,[59–61] as demonstrated in open-angle glaucoma and ocular hypertension patients[62] by measurement of corneal autofluorescence.[47] As shown in Fig. 10, the corneal epithelial permeability and thus the autofluorescence values of patients using timolol as well as those of patients not using timolol were significantly higher than those of healthy controls (mean increase, 40 and 21%, respectively; Mann-Whitney test, $P = 0.002$).

The 40 and 21% increase in the values of corneal autofluorescence in open-angle glaucoma or ocular hypertension patients compared with those of healthy controls indicate, as Van Best stated, an increase in corneal metabolism associated with the disease, but apparently not with the daily instillation of timolol. The reasons for the alterations in corneal metabolism that occur in glaucoma remain speculative. One explanation might be the appearance of an autonomic neuropathy, which has been suggested as an etiological factor in the pathogenesis of glaucoma.[63–66] An autonomic neuropathy may change corneal innervation and consequently alter corneal metabolism.

In penetrating keratoplasty corneas, the corneal metabolism can be influenced by the surgical denervation of the cornea.[67–69] This may contribute to the changes measured in corneal autofluorescence since other factors such as recipient's age, donor age, and total lifespan of the transplant did not influence the corneal autofluorescence.[47]

4.2 *Lens Autofluorescence*

Studies on the transmission and autofluorescence properties of ocular media have been performed using ASF. The transmission of the lens in relation to aging and pathology is being consistently studied by several groups.[70–72]

As shown in Fig. 11, the fluorescence of the lens has been found to increase linearly with age in healthy individuals.[73] Strong indications were found that the UV or UVA radiation in sunlight was responsible for this increase.[74–76] With age, transmission of blue light decreases, whereas the transmission of green to red light is less modified.

The correlation between autofluorescence of the human lens and diabetes mellitus has been investigated over the past three decades.[77] One of the most provocative results of this technique is its apparent ability to detect a prediabetic condition when no other symptoms are present. Lens autofluorescence in diabetic patients was found to be related to metabolic regulation[78] and to increase twice as fast as that in healthy individuals.[79] The correlation between the fluorescence ratio and the invasive hemoglobin A1C test results has been demonstrated. Unfortunately a large dependence of the lens autofluorescence on the subject's age has been observed and therefore an age correction for the data obtained from autofluorescence measurements is essential. This correction is critical since the model used to describe the dependence of the fluorescence ratio and age introduces a statistical spread of the measured data.

The initial stages of cataract are characterized by the appearance of lipid fluorophores in the near-ultraviolet and violet regions of the spectrum, whereas in advanced stages an increase in the intensity of the long-wave fluorescence of the lipids in the blue-green region occurs.[80] It has been proposed that the decreased transmission of visible light in the aging lens and in nuclear cataract is mainly due to the accumulation

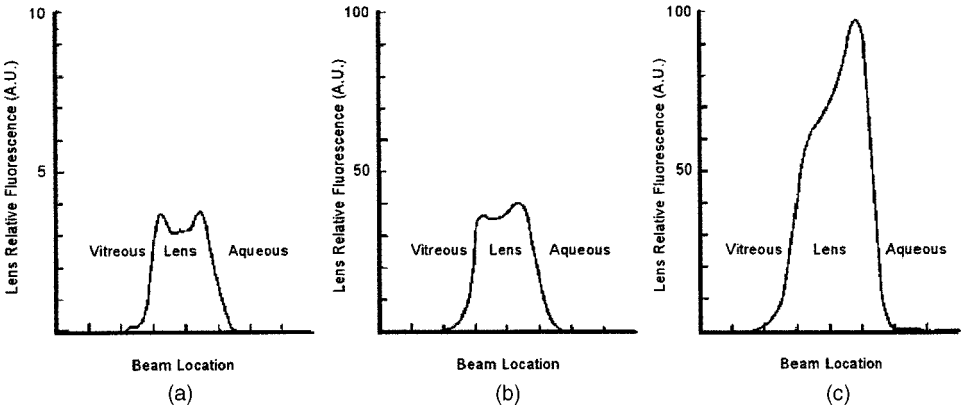

Fig. 11 Typical lens autofluorescence curves of healthy individuals aged (a) 7 years, (b) 53 years, and (c) 84 years. Maximum autofluorescence increases and lens transmission index decreases with age. Note different scale in (a) (Reproduced from Ref. 73 with permission.)

of lens fluorogens rather than configurational changes of the lens proteins, as seen in cortical cataracts. The lens fluorogens are responsible, to a significant degree, for the increasing yellow color of the lens with age. The nuclear and perinuclear cortical regions of the lens show the most intense blue-green autofluorescence, which may be derived from a UV light-induced tryptophan photodegradation reaction or caused by nonenzymatic glycosylation of the lens proteins.

In cataractous lenses, the mean autofluorescence and scatter values are statistically significantly different from those of age-matched healthy controls. Many investigators have proposed a UV light-induced photo-oxidative mechanism for cataract formation. In part, the low glutathione concentration in the lens nucleus makes it particularly vulnerable to long-term photo-oxidative stress following crystallin aggregation, pigmentation, and development of nontryptophan fluorescence. Measurements of corneal and lens autofluorescence will be of particular interest because of their clear association with morphological and biochemical changes in these tissues.[57]

4.3 *Autofluorescence of Retinal Pigment Epithelium*

Lipofuscin is a fluorescent pigment that is absent in fetal and newborn RPEs; it continuously accumulates in the RPE as a result of incomplete digestion of spent rod outer segment disks.[81–86] An increase of about 40% in the fluorescence intensity of lipofuscin is observed with increasing age.[86] Therefore lipofuscin is commonly considered as an indicator of aging. Retinal pathologies such as age-related maculopathy (ARM), senile macular degeneration (SMD), retinitis pigmentosa (RP), and retinal abnormalities related to the Sjögren-Larsson syndrome (SLS), all exhibit abnormal content and distribution of lipofuscin.[82,87–88]

Spaide[89] recently showed that there is no correlation between autofluorescence measurements and the degree of nuclear sclerosis ($P=0.14$), whereas patients with exudative AMD had more autofluorescence in the fellow eye than did patients without a history of exudative AMD ($P=0.002$). Patients with retinal vascular anastomosis have focal areas of intense autofluorescence in their fellow eye that corresponded, for the most part, to focal areas of hyperpigmentation best seen by infrared monochromatic fundus photography ($P=0.015$). These findings suggest possible explanations for certain patterns of vessel growth seen in exudative AMD.

RPE autofluorescence was used to study lipofuscin distribution associated with individual drusen in patients with ARM or SMD.[90] A specific pattern of autofluorescence was frequently found to be spatially associated with hard drusen and soft drusen between 60 and 175 μm in size. The pattern is characterized by a central area of decreased autofluorescence surrounded, in most cases, by an annulus of increased autofluorescence. The location of this pattern was highly correlated with the position of individual distinct drusen. The central low autofluorescence focus was on average 16% below the surrounding background, and the annulus, when present, was on average 6% more fluorescent than the background.

5 Conclusions and Outlook

Ocular fluorometry, alone or in combination with other optical methods of investigation of the ocular media, has reached full maturity, and this is due primarily to considerable efforts in the investigation of fluorophores and their relation to age and pathologies in the eye, and to the dramatic improvement in optical and electro-optical components and devices. The high social relevance of diagnostic instruments for population screening, and the increase in the average age of a modern population (stressing the importance of careful treatment of age-related pathologies to maintain an adequate quality of life in the elderly) are indicators of the fact that the near future will bring us new, sophisticated instruments for rapid, timely, noninvasive diagnoses in ophthalmology where fluorometry can play a significant role.

References

1. L. I. Grossweiner, ''Photophysics,'' in *The Science of Photobiology*, K. C. Smith Ed., Chap. 1, Plenum, New York (1989).
2. Z. I. Vavilov, ''Die fluoreszenzausbeute von farbstofflösungen,'' *Z. Phys.* **22**, 266 (1924).
3. M. Kasha, ''Characterization of electronic transitions in complex molecules,'' *Discuss. Faraday Soc.* **9**, 14 (1950).
4. J. R. Kincaid, in *Instrumental Analysis*, G. D. Christian and J. E. O'Reilly Eds., Chap. 8, Allyn and Bacon, Boston (1986).
5. H. Du, R. A. Fuh, J. Li, A. Corkan, and J. S. Lindsey, ''Photochem-

CAD: a computer-aided design and research tool in photochemistry,'' *Photochem. Photobiol.* **68**, 141–142 (1998).
6. R. B. Kurzel, M. L. Wolbarsht, and B. S. Yamanashi, ''Spectral studies on normal and cataractous intact human lenses,'' *Exp. Eye Res.* **17**, 65–71 (1973).
7. J. A. van Best, J. L. van Delft, and J. E. Keunen, ''Long term follow-up of lenticular autofluorescence and transmittance in healthy volunteers,'' *Exp. Eye Res.* **66**, 117–123 (1998).
8. S. Lerman, J. F. Kuck, J. F. Borkman, and E. Saker, ''Induction, acceleration and prevention (*in vitro*) of a parameter in the ocular lens,'' *Ophthalmic. Res.* **8**, 213–226 (1976).
9. F. Docchio and L. Biancardi Eds., *EUROEYE Atlas of Ocular Fluorophores*, Grafica de Coimbra, Coimbra, Portugal (1995).
10. S. Udenfrend, *Fluorescence Assays in Biology and Medicine*, Vol. II, Academic Press, New York (1969).
11. S. Granhee and V. M. Monnier, ''Mechanism of formation of the Maillard protein cross-link pentosidina. Glucose, fructose and ascorbate as pentosidine precursors,'' *J. Biol. Chem.* **266**, 11649–11653 (1991).
12. R. Jacobs and D. L. Krohn, ''Fluorescence intensity profile of human lens sections,'' *Invest. Ophthalmol. Visual Sci.* **20**, 117–120 (1981).
13. P. P. Fagerholm, B. T. Philipson, and B. Lindstrom, ''Normal human lens—the distribution of proteins,'' *Exp. Eye Res.* **33**, 615–620 (1981).
14. G. J. H. Bessems, E. Keizer, J. Wollensak, and H. J. Hoenders, ''Non-tryptophan fluorescence of crystallins from normal and cataractous human lens,'' *Invest. Ophthalmol. Visual Sci.* **28**, 1157–1163 (1987).
15. H. J. Hoenders and H. Bloemendal, ''Aging of lens proteins,''in *Molecular and Cellular Biology of the Eye Lens*, H. Bloemendal, Ed., pp. 273–326, Wiley, New York (1981).
16. R. van Heyningen, ''The glucoside of 3-hydroxykynurenine and other fluorescent compounds in the human lens,'' in *The Human Lens in Relation to Cataract*, pp. 151–168, Ciba Found. Symp. Vol. 19, Elsevier, New York (1973).
17. M. Bando, N. T. Yu, and J. F. R. Kuck, ''Fluorophors and chromophors from rat lens crystallins in UV with hydroxykynurenine,'' *Invest. Ophthalmol. Visual Sci.* **25**, 581–585 (1984).
18. J. A. van Best and E. V. M. J. Kuppens, ''Summary of studies on the blue-green autofluorescence and light transmission of the ocular lens,'' *J. Biomed. Opt.* **1**, 243–250 (1996).
19. N. T. Yu, M. Bando, and J. F. R. Kuck, ''Metabolic production of a blue-green fluorophor in lenses of dark-adapted mice and its increase with age,'' *Invest. Ophthalmol. Visual Sci.* **24**, 1157–1161 (1983).
20. N. T. Yu, D. J. Y. Ho, and J. F. Kuck Jr., ''Automated laser-scanning microbeam fluorescence/Raman image analysis of human lenses with multichannel detection: evidence for metabolic production of a green fluorophor,'' *Proc. Natl. Acad. Sci. U.S.A.* **85**, 103–106 (1988).
21. B. C. Barron, N. T. Yu, and J. F. R. Kuck, ''Distribution of a 488 nm excited fluorophor in the equatorial plane of human lens by a laser Raman microprobe: a new concept in fluorescence studies,'' *Exp. Eye Res.* **47**, 901–904 (1988).
22. F. Docchio, Ed., *Introduction to Ocular Fluorometry*, Grafica de Coimbra, Coimbra Portugal (1997).
23. R. Cubeddu, F. Docchio, R. Ramponi, and M. Boulton, ''Time-resolved fluorescence spectroscopy of the retinal pigment epithelium: age-related studies,'' *IEEE J. Quantum Electron.* **26**, 2218–2225 (1990).
24. M. Boulton, F. Docchio, P. Dayhaw-Barker, R. Ramponi, and R. Cubeddu, ''Age-related changes in the morphology, absorption and fluorescence of melanosomes and lipofuscin granules of the retinal pigment epithelium,'' *Vision Res.* **9**, 1291–1303 (1990).
25. R. C. Zeimer, N. P. Blair, and J. G. Cunha-Vaz, ''Vitreous fluorophotometry for visual research: 1. Description and evaluation of a new fluorophotometer,'' *Arch. Ophthalmol.* **101**, 1753–1756 (1983).
26. F. Docchio, ''Ocular fluormetry: principle, fluorophores, instrumentation, and clinical applications,'' *Lasers Surg. Med.* **9**, 515–532 (1989).
27. O. Hockwin, V. Dragomirescu, and H. Laser, ''Age-related changes in normal and cataractous human lens density estimated by microdensitometric image analysis of Scheimpflug photographs,'' *Lens Res.* **1**, 207–220 (1983).
28. V. Dragomirescu, O. Hockwin, and H. R. Koch, ''Development of a new equipment for rotating slit image photography according to Scheimpflug's principle,'' *Interdiscipl. Topics Geront.* **13**, 118–130 (1978).
29. W. Fink, H. J. Huebscher, and T. Seiler, ''Correction of biometric data derived from digital Scheimpflug records,'' *Proc. ARVO IOVS* **39**, 1033 (1998).
30. O. Hockwin, V. Dragomirescu, H. Laser, A. Wegener, and U. Eckerskorn, ''Measuring lens transparency by Scheimpflug photography of the anterior eye segment: principle, instrumentation and application to clinical and experimental ophthalmology,'' *J. Toxicol. Cut. Ocular Toxicol.* **6**, 251–271 (1987).
31. S. Lerman and O. Hockwin, ''Ultraviolet-visible slit lamp densitography of the human eye,'' *Exp. Eye Res.* **33**, 587–596 (1981).
32. S. Lerman, ''*In vivo* and *in vitro* biophysical studies on human cataractogenesis,'' *Lens Res.* **3**, 137–160 (1986).
33. S. Lerman, ''Chemical and physical properties of the normal and aging lens: spectroscopic (UV, fluorescence, phosphorescence, NMR) analyses,'' *Am. J. Optom. Physiol. Opt.* **64**, 11–22 (1987).
34. K. Kashima, B. L. Trus, M. Unser, M. B. Datiles, and P. A. Edwards, ''Aging studies of normal volunteer lens using the Scheimpflug slit lamp camera,'' *Invest. Ophthalmol. Visual Sci.* **30** (Suppl.), 164 (1989).
35. O. Hockwin, V. Dragomirescu, and B. Schwarz, ''Monitoring lens transparency during in vitro incubation by Scheimpflug photography and densitometric image analysis,'' *Ophthalmic Res.* **17**, 96–101 (1985).
36. N. Lois, A. S. Halfyard, and A. C. Bird, ''Quantitative evaluation of fundus autofluorescence imaged *in vivo* in eyes with retinal disease,'' *Br. J. Ophthamol.* **84**, 741–745 (2000).
37. N. M. Haralampus-Grynaviski, L. E. Lamb, J. D. Simon, J. R. Krogmeier, R. C. Dunn, A. Pawlak, M. Rózanowska, T. Sarna, and J. M. Burke, ''Probing the spatial dependence of the emission spectrum of single human retinal lipofuscin granules using near-field scanning optical microscopy,'' *Photochem. Photobiol.* **74**, 364–368 (2001).
38. J. N. Miller, ''Stray light in fluorescence spectrometers,'' in *Standards in Fluorescence Spectrometry*, J. N. Miller Ed., Chap. 8, Chapman and Hall, London (1981).
39. C. A. Parker and W. T. Rees, ''Fluorescence spectrometry. a review,'' *Analyst (Cambridge, U.K.)* **87**, 83 (1962).
40. C. R. Munnerlyn, J. R. Gray, and D. R. Hennings, ''Design considerations for a fluorophotometer for ocular research,'' *Graefe's Arch. Clin. Exp. Ophthalmol.* **222**, 209–211 (1985).
41. J. A. Van Best and F. Docchio, ''Simple, low-cost, portable corneal fluorometer for detection of the level of diabetic retinopathy,'' *Appl. Opt.* **37**, 4303–4311 (1998).
42. American National Standards Institute, *Safe Use of Lasers* ANSI standard **Z136.1**, ANSI, New York (1993).
43. W. Lohmann, W. Schmehl, P. Bernhardt, H. Wickert, M. Ibrahim, and J. Strobel, ''Device for measuring native fluorescence of lenses,'' *J. Biochem. Biophys. Methods* **17**, 155–158 (1988).
44. W. Lohmann, M. Wunderling, W. Schmehl, J. Strobel, and K. G. Bensch, ''Nuclear cataract and ascorbic acid,'' *Naturwissenschaften* **73**, 266–267 (1986).
45. B. Chu, *Laser Light Scattering*, Academic Press, New York (1974).
46. L. Rovati, L. Pollonini, and R. R. Ansari, ''An integrated instrument for dynamic light scattering and natural fluorescence measurements,'' *Proc. SPIE* **4245**, 192–198 (2001).
47. Th. R. Stolwijk, J. A. van Best, J. P. Boot, and J. A. Oosterhuis, ''Corneal autofluorescence in diabetic and penetrating keratoplasty patients as measured by fluorophotometry,'' *Exp. Eye Res.* **51**, 403–409 (1990).
48. M. Larsen, B. Kjer, I. Bendtson, P. Dalgaard, and H. Lund-Andersen, ''Lens fluorescence in relation to metabolic control of insulin-dependent diabetes mellitus,'' *Arch. Ophthalmol.* **107**, 59–62 (1989).
49. B. R. Master, ''Noninvasive redox fluorometry: how light can be used to monitor alterations of corneal mitochondrial function,'' *Curr. Eye Res.* **3**, 23–26 (1984).
50. V. M. Monnier, V. Vishwanath, K. E. Frank, C. A. Elmets, P. Dauchot, and R. P. Kohn, ''Relation between complication of type I diabetes mellitus and collagen-linked fluorescence,'' *N. Engl. J. Med.* **314**, 403–408 (1986).
51. P. R. Odetti, A. Borboglio, and R. Ronaldi, ''Age-related increase of collagen fluorescence in human subcutaneous tissue,'' *Metabolism* **41**, 655–658 (1992).
52. V. M. Monnier, R. P. Kohn, and A. Cerami, ''Accelerated age-related browning of human collagen in diabetes mellitus,'' *Proc. Natl. Acad. Sci. U.S.A.* **81**, 583–587 (1984).
53. Th. R. Stolwijk, J. A. van Best, J. A. Oosterhuis, and W. Swart, ''Corneal autofluorescence: an indicator of diabetic retinopathy,'' *Invest. Ophthalmol. Visual Sci.* **33**, 92–97 (1992).

54. S. Fantaguzzi, F. Docchio, L. Guarisco, and R. Brancato, ''Corneal autofluorescence in diabetic and normal eyes,'' *Int. Ophthalmol.* **18**, 211–214 (1995).
55. L. Rovati, F. Docchio, C. Azzolini, and Jaap Van Best, ''Corneal autofluorescence in presence of diabetic retinopathy,'' *Proc. SPIE* **3246**, 22–27 (1998).
56. J. Steinke and J. S. Soeldner, ''Diabetes mellitus,'' in *Principles of Internal Medicine*, G. W. Thorn, R. D. Adams, E. Braunwald, K. J. Isselbacher, and R. G. Petersdorf, Eds., pp. 563–583, McGraw-Hill, Kogakusha, Tokyo (1987).
57. L. Rovati, F. Fankhauser II, F. Docchio, and J. Van Best, ''Diabetic retinopathy assessed by dynamic light scattering and corneal autofluorescence,'' *J. Biomed. Opt.* **3**(3), 357–363 (1998).
58. J. N. Lian and L. J. Chylack, ''Change in the protein tertiary structure with non-enzymatic glycosylation of calf alpha crystalline,'' *Biochem. Biophys. Res. Commun.* **123**, 899–906 (1984).
59. F. T. Fraunfelder, ''Drugs used primarily in ophthalmology,''in *Drug-Induced Ocular Side Effects and Drug Interactions*, S. M. Meyer, Ed., pp. 476–477, Lea & Febiger, Philadelphia, PA (1989).
60. J. A. M. Ramselaar, J. P. Boot, N. J. van Haeringen, J. A. van Best, and J. A. Oosterhuis, ''Corneal epithelial permeability after instillation of ophthalmic solutions containing local anaesthetics and preservatives,'' *Curr. Eye Res.* **7**, 947–950 (1988).
61. N. L. Burstein, ''Preservative alteration of corneal permeability in human and rabbits,'' *Invest. Ophthalmol. Visual Sci.* **25**, 1453–1457 (1984).
62. E. Kuppens, Th. Stolwijk, J. A. van Best, and de R. Keizer, ''Topical timolol, corneal epithelial permeability and autofluorescence in glaucoma by fluorophotometry,'' *Graefe's Arch. Clin. Exp. Ophthalmol.* **232**, 215–220 (1994).
63. C. V. Clark, ''Autonomic denervation hypersensitivity in the primary glaucomas,'' *Eye* **3**, 349–354 (1989).
64. K. Jordan, S. R. Perry, P. J. McCormack, and R. N. Downes, ''Cholinergic supersensitivity of the iris in primary open-angle glaucoma, A clinical study,'' *Eye* **2**, 233–237 (1988).
65. E. J. F. M. de Kruijf, J. P. Boot, L. Laterveer, J. A. van Best, J. A. M. Ramselaar, and J. A. Oosterhuis, ''A simple method for determination of corneal epithelial permeability in humans,'' *Curr. Eye Res.* **6**, 1327–1334 (1987).
66. D. F. Sweeney, A. Vannas, B. A. Holden, T. Tervo, and T. Telaranta, ''Evidence for sympathetic neural influence on human corneal epithelial function,'' *Acta Ophthalmol.* **63**, 215–220 (1985).
67. R. S. Chuck, M. E. Arnoldussen, A. Behrens, P. M. Sweet, G. H. Bearman, and W. S. Grundfest, ''Fluorescence-guided laser removal of chemically damaged cornea,'' *J. Cataract Refractive Surg.* **28**, 1847–1852 (2002).
68. M. N. Ediger, D. W. Hahn, and G. H. Pettit, ''Characterization of products of excimer laser ablation of collagen,'' *Proc. SPIE* **2393**, 106–110 (1995).
69. S. Tuft, R. Al-Dhahir, P. Dyer, and Z. Zehao, ''Characterization of the fluorescence spectra produced by excimer laser irradiation of the cornea,'' *Invest. Ophthalmol. Visual Sci.* **31**, 1512–1518 (1990).
70. J. R. Evans, A. Rauf, A. A. Sayer, R. P. L. Wormald, and C. Cooper, ''Age-related nuclear lens opacities are associated with reduced growth before 1 year of age,'' *Invest. Ophthalmol. Visual Sci.* **39**, 1740–1744 (1998).
71. G. Duncan, I. M. Wormstone, and P. D. Davies, ''The aging human lens: structure, growth and physiological behaviour,'' *Br. J. Ophthamol.* **81**, 818–823 (1997).
72. A. Spector, S. Li, and J. Sigelman, ''Age dependent changes in molecular size of lens proteins and their relationship to light scatter,'' *Invest. Ophthalmol. Visual Sci.* **13**, 795–798 (1974).
73. S. Siik, ''Lens autofluorescence in aging and cataractous human lenses. Clinical applicability,'' *Acta Universitatis Ouluensis Medica* **D 525** (1999).
74. J. Dillon, ''UV-B as a pro-aging and pro-cataract factor,'' *Doc. Ophthalmol.* **88**, 339–344 (1995).
75. U. P. Andley and B. A. Clark, ''Generation of oxidants in the near-UV photooxidation of human lens a-crystallin,'' *Invest. Ophthalmol. Visual Sci.* **30**, 706–713 (1989).
76. S. Zigman, G. Geiss, T. Yulo, and J. Schultz, ''Ocular protein alteration by near UV light,'' *Exp. Eye Res.* **15**, 255–264 (1973).
77. M. Bando and A. Nakajima, ''Fluorescence in human lens,'' *Exp. Eye Res.* **16**, 167 (1973).
78. S. Ishiko, A. Yoshida, F. Mori, T. Abiko, N. Kitaya, S. Konno, and Y. Kato, ''Corneal and lens autofluorescence in young insulin-dependent diabetic patients,'' *Ophthalmologica* **212**, 301–305 (1998).
79. J. C. Bleeker, J. A. van Best, L. Vrij, E. A. van der Velde, and J. A. Oosterhuis, ''Autofluorescence of the lens in diabetic and healthy subjects by fluorophotometry,'' *Invest. Ophthalmol. Visual Sci.* **27**, 791–794 (1986).
80. M. A. Babizhayev, ''Lipid fluorophores of the human crystalline lens with cataract,'' *Graefe's Arch. Clin. Exp. Ophthalmol.* **227**, 384–391 (1989).
81. G. E. Eldred and M. L. Katz, ''Fluorophores of the human retinal pigment epithelium: separation and spectral characteristics,'' *Exp. Eye Res.* **47**, 71–86 (1988).
82. P. Rakoczy, C. Kennedy, D. Thompson-Wallis, K. Mann, and I. Constable, ''Changes in retinal pigment epithelial cell autofluorescence and protein expression associated with phagocytosis of rod outer segments *in vitro*,'' *Biol. Cell* **76**, 49–54 (1992).
83. M. Boulton and J. Marshall, ''Effects of increasing numbers of phagocytic inclusions on human retinal pigment epithelial cells in culture: a model for aging,'' *Br. J. Ophthamol.* **70**, 808–815 (1986).
84. D. Bok, ''Retinal photoreceptors—pigment epithelium interactions,'' *Invest. Ophthalmol. Visual Sci.* **26**, 1659–1694 (1985).
85. G. E. Eldred, G. V. Miller, G. V. Stark, and L. Feeney Burns, ''Lipofuscin: resolution of discrepant fluorescence data,'' *Science* **216**, 757–759 (1982).
86. M. Boulton, F. Docchio, R. Cubeddu, R. Ramponi, and P. D. Barker, ''Age-related changes in the fluorescence of melanin and lipofuscin granules of retinal pigment epithelium: a time-resolved fluorescence spectroscopy study,'' *Photochem. Photobiol.* **54**, 247–253 (1991).
87. A. von Rückmann, W. F. Fitzke, and A. C. Bird, ''Clinical investigation: distribution of pigment epithelium autofluorescence in retinal disease state recorded *in vivo* and its change over time,'' *Graefe's Arch. Clin. Exp. Ophthalmol.* **237**, 1–9 (1999).
88. S. E. G. Nilsson and S. Jagell, ''Lipofuscin and melanin content of the retinal pigment epithelium in a case of Sjoegren-Larsson syndrome,'' *Br. J. Ophthamol.* **71**, 224–226 (1987).
89. R. F. Spaide, ''Fundus autofluorescence and age-related macular degeneration,'' *Ophthalmology* **110**, 392–399 (2003).
90. F. C. Delori, M. R. Fleckner, D. G. Goger, J. Weiter, and C. K. Dorey, ''Autofluorescence distribution associated with drusen in age-related macular degeneration,'' *Invest. Ophthalmol. Visual Sci.* **41**, 496–504 (2000).