Simple, low-cost, portable corneal fluorometer for detection of the level of diabetic retinopathy

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A simple, low-cost, portable instrument for measurement of the autofluorescence of the human cornea is presented. Corneal autofluorescence has proved to be strongly correlated with the grade of retinopathy in diabetic patients. It is therefore a reliable parameter for detection of different levels of diabetic retinopathy, thus permitting timely intervention by ophthalmologists. The instrument contains custom optics and electronics and exhibits excellent linearity and repeatability both in vitro and in vivo. Preliminary tests on volunteers show promise for its use in clinical practice. © 1998 Optical Society of America

1. Introduction

The detection of the level of diabetic retinopathy (DR) in diabetic patients is of crucial interest in health care, as this type of ocular disease is one of the main causes of blindness in the Western world. Detecting severe stages of DR in time, in most cases, postpones or even prevents the onset of blindness through a timely start of laser therapy.

Screening for DR in clinical practice is generally performed by biomicroscopy of the eye fundus. Ophthalmoscopy performed under optimal conditions by internists, senior medical residents, and diabetologists has resulted in a percentage of missed diagnoses of (pre)proliferative retinopathy, the most severe stage of retinopathy, of 52%, 50%, and 33%, respectively. For the more advanced levels of retinal pathology, fluorescein angiography of the retina is used. Both techniques are only qualitative and must be administered by an ophthalmologist specialized in diabetic retinopathy. Moreover, the latter technique is invasive because it makes use of fluorescent markers such as disodium fluorescein (NaFl), which is applied intravenously. Another objective screening technique for DR is automated retinal image documentation. This method is still under development and seems promising. However, the technique requires digitized ocular fundus images.

Some other techniques for screening of diabetic retinopathy are based on the measurement of local changes in the vitreous viscosity close to the retina that occur as a consequence of the disease. These measurements are performed by dynamic light scattering techniques and laser radiation and are still performed only at the laboratory level. The use of a laser focused close to the retina may be a drawback in terms of safety of the procedure in clinical practice.

Recently it was shown that the autofluorescence of the corneal tissue within specific wavelength regions is sensibly increased in the presence of DR. This fact is of particular relevance because the cornea is readily accessible to investigation and because corneal autofluorescence, in contrast to lens autofluorescence, is not age related in healthy persons.

Although the nature and the exact excitation and emission wavelength ranges of the fluorophore(s) of interest, especially those responsible for the increased corneal autofluorescence in patients with DR, are still unknown and are subjects for investigation, the use of corneal autofluorescence as an indicator of DR in different stages is promising, as has been demonstrated by our colleagues as well as by others using a commercially available instrument (Fluorotron Master, Ocumetrics, Inc., Mountain View, Calif.). Indeed, corneal autofluorescence has proved to be a better indicator of the state of the retina than is the duration of the diabetes: In fact, mean corneal autofluorescence was 30% higher in patients with (pre)proliferative retinopathy than...
in patients with background retinopathy, whereas the duration of diabetes in both groups was not significantly different. The sensitivity for detecting DR in diabetes patients (i.e., the percentage of positive diagnoses in all eyes that have retinopathy) by the use of corneal autofluorescence was 80%, and the specificity (i.e., the percentage of negative diagnoses in all eyes that did not have the disease) was 76%.

In a recent 3-year multicenter study (European Concerted Action on Ocular Fluorometry, Clinical component BMH1-CT92-0477), sensitivity and specificity of 100% and 68%, respectively, for detecting (pre)proliferative retinopathy by corneal autofluorescence were found. A sensitivity of 100% indicates that no patient in danger of losing vision if no appropriate measures were taken was misdiagnosed. In the same study, corneal autofluorescence was found to be also predictive of the need for laser treatment within 1 year after measurement, with a sensitivity of 100% and a specificity of 68%.

In vivo measurements with a fluorophotometer with a set of adjustable excitation wavelengths revealed an excitation region at 440–470 nm, which could probably be attributed to flavin mononucleotide. Changes in the metabolism of mitochondrial flavoproteins can be held responsible for the increased corneal autofluorescence in diabetic patients because (i) the autofluorescence of these proteins depends on metabolic changes in glucose levels, oxygen availability, and the mitochondrial respiratory state and (ii) the fluorescence excitation and emission wavelengths (λ = 460 nm and λ = 540 nm, respectively) correspond to the excitation and detection wavelengths of the fluorophotometer used for measurements in diabetic patients.

Following the initial investigations it was thought that a simple, compact, and low-cost instrument designed to measure changes in corneal autofluorescence could be of great interest for public health care. Such a system would have a large effect in the prevention of blindness as a result of DR because its low cost and simplicity of use would facilitate its use not only by ophthalmologists but also by general practitioners. It would make early monitoring of the progression of DR possible in many patients and consequently permit timely intervention (such as laser treatment) by an ophthalmologist to stop the progression of the disease.

We present a novel measuring apparatus developed in our laboratories for the measurement of corneal autofluorescence. The system measures the autofluorescence of the cornea over an excitation and emission range used in previous measurements. In its present version the prototype 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. Note that lens autofluorescence values of healthy persons are strongly age dependent, starting from near zero value at birth and increasing linearly to a value of approximately 50 times that of the cornea at the age of 90. Furthermore, the lenticular values have a large interindividual variability (greater than 50%) in healthy individuals of the same age, whereas corneal fluorescence is age independent with an interindividual variability of 25%.

To reduce costs we used custom light-emitting diodes (LED’s) in combination with suitable, inexpensive color filters as the excitation source. Custom signal-conditioning electronics were developed to treat the signal for subsequent conversion. A single-chip microprocessor unit was designed to perform the control of the acquisition, to convert the acquired signal into digital form, and to display the results. To add flexibility in the initial phase of the instrument characterization, a virtual version of the instrument running on a PC was implemented.

In this paper we describe the instrument in detail and report on its characterization and on a preliminary session of tests on volunteers to assess its potential.

2. Description of the System

A. Introduction

We describe the portable corneal fluorometer with reference to the general block diagrams of Fig. 1 and to the to-scale details in Fig. 2. The instrument is composed [Fig. 1(a)] of a modified photographic camera (Pentax K1000 reflex camera; lens 50 mm, f/2) equipped with a flexible bellows and connected to an electronic unit. The purpose of the camera is to make an image of the central part of the cornea on the sensitive area of a photomultiplier tube (PMT). A special adapter is mounted onto the camera lens to include all light sources for the excitation of the fluorescence plus additional light sources that are described below. All light sources in the instrument are low-power LED’s.

B. Portable Optical Head

1. Blue Light

The optical head of the portable corneal fluorometer is described with reference to Fig. 1(b). The corneal excitation light sources consist of six LED’s emitting blue light [Kingbright Model L 934BT; 7–14 millican-dells (med); a candela is the SI base unit of luminous intensity; see Table 1]. The outgoing LED light (Fig. 2), which is emitted parallel to the optical axis of the camera, passes through a set of three wideband interference filters that select only the wavelength region of interest for corneal excitation (ODL Model D-Blue-SW490).

The filtered LED light is deflected by a total internal reflection prism that diverts it by 90° and is used to illuminate the cornea tangentially at the left and right sides of the eye. In the instrument the patient-oriented edges of the prisms are rounded so
would not hurt the patient. Exciting the cornea tangentially is necessary for the following reason: The fluorescence emitted by the cornea is weak compared with the much higher fluorescent emission of the underlying crystalline lens, with largely overlapping excitation and emission spectra. Therefore the tangential excitation has the purpose of minimizing the amount of excitation light that reaches the lens. The central part of the blue LED beam is used to excite the sample volume of the cornea [Fig. 2(a)]. The light power in that part was found to be homogeneous within 30%. Parts of the prisms are covered to prevent blue light from illuminating other parts of the eye or its environment.

2. Fluorescence
The fluorescence emitted by the corneal tissue in response to the blue excitation light passes through a suitable set of barrier filters [Fig. 1(b); two blue light blocking filters (Schott Model OG515) and one IR radiation blocking filter (Schott Model BG18) with a transmittance of less than $10^{-5}$ at $\lambda = 900–1000$ nm] and is imaged by the camera lens onto the photosensitive area ($4 \, \text{mm} \times 13 \, \text{mm}$) of a miniature photomultiplier with built-in power supply (Hamamatsu Model HC120) fixed at the back of the camera. The linear magnification factor of the camera lens is 0.8. The power supply of the photomultiplier is stabilized by means of an external reference voltage generator.

A standardized part of the corneal surface ($5 \, \text{mm} \times 10 \, \text{mm}$; Fig. 2(b)) is selected by means of a fixed rectangular aperture in front of the PMT’s sensitive area [Fig. 1(b)]. Consequently the sampled fluorescence volume consists of a rectangular part of the cornea with a round hole of 3-mm diameter in the middle (Fig. 2). The hole is due to shadowing by the
red LED, which is positioned above the center of the cornea. The sample volume of the cornea was calculated by three-dimensional numerical integration in a cornea with an outer radius of 7.8 mm, an inner radius of 6.8 mm, and a central thickness of 0.525 mm (Ref. 18) and amounted to 28.9 mm³. Increasing or decreasing the outer corneal radius by 10% while holding the other parameters constant resulted in a change of calculated volume of 12%. Note that corneal autofluorescence is increased by 50% to 100% in patients with (pre)proliferative retinopathy. 8

3. Optical Filters

Figure 3 (upper curves) shows the spectral transmission curves of the excitation and emission filters, the relative blue LED light power, and the light power that results after the light has passed through the excitation filters. For fluorescence measurements it is important that the excitation and the emission spectral regions have minimum overlap to prevent any contribution of the excitation light from reaching the detector. This overlap has been minimized as shown in Fig. 3 (lowest curve), where the overlap between the two filter combinations (three excitation filters and two emission filters placed in sequence) is shown. It can be seen that the combined transmittance is less than 10⁻⁸ in the total wavelength range. Such low transmittance is essential, given the low intensity of corneal autofluorescence in comparison with the high intensity of the excitation light reflected by the corneal surface. A signal originating from reflection of the excitation light of less than 13% of the corneal autofluorescence signal was obtained with the use of a similar filter combination. 10 Note that the reflected signal consists of blue excitation light from the blue LED’s that still passes through the emission filters as well as green light emitted by the blue LED’s that still passes through the excitation filters.

4. Green Light

In addition to the blue light sources, 10 green LED’s are mounted upon a ring on the illumination adapter [Figs. 1(b) and 2(b)]. These are high efficiency LED’s (Table 1: 200–300 mcd; Kingbright Type L934SGC/B) that help the operator to position the eye correctly within the field of view of the reflex camera objective, because green light passes through
the emission filters. An additional purpose of these green LED's is to force the constriction of the iris of the target eye as an extra means to reduce excitation of fluorescence of the ocular lens. The green LED's are turned off immediately before measurement of the green fluorescence is started.

5. Red Light
A third light source is used in the apparatus. It is a single high-efficiency red LED (Table 1: 700–1000 mcd; Kingbright Type L934SR) mounted at the center of the adapter on the optical axis of the system. This light source has three purposes. The first is to serve as a fixation aid for the patient before and during the measurement, so that the eye is oriented correctly with respect to the optical axis. The second purpose is to keep the pupil constricted during the measurement to reduce interference by the crystalline lens fluorescence (see Subsection 2.B.6). The third is to prevent central fluorescent emission, which originates from the crystalline lens, from reaching the photodetector in a direct way [by shadowing; see Figs. 1(b) and 2(a)].

6. Infrared Light
Finally, the optical head is equipped with an IR transmitter–receiver combination (IR LED; Table 1: Kingbright Type L934F3C, half-width 925–970 nm). The transmitter is located inside one of the two illuminators, and the receiver (photodiode) is housed in the other one. Note that the excitation filter combination is transparent to IR light (transmittance better than 60% between 800 and 1100 nm). The transmitter is square-wave modulated: The receiver signal, suitably shaped, drives an earphone set that the operator wears during the measurement. (In addition, it operates a small red LED in the camera viewer.)

When the cornea is in place at the correct distance from the camera lens the transmitter–receiver path is on the border of being interrupted and the earphone signal becomes inaudible, thus indicating that the system is ready for operation. In this position the cornea and the rectangular aperture in front of the PMT are on conjugate planes. By this procedure the autofluorescence of a fixed volume of the cornea is measured (Fig. 2). An IR-blocking filter (ODL type SW-710) is placed in the emission path to prevent interference of the fluorescence signal.

C. Safety Considerations
From the above description it follows that four types of light source (blue, green, red, and IR low-power LED’s) are used in the instrument. We calculated the total light energy entering the eye for each type, taking into account the total emitted power, angles of emission, transmittance of the excitation filters and prisms, angles of incidence, and distances between light sources and the eye, under the assumption of a constricted pupil diameter of 2 mm (Ref. 19) and a focusing time of 3s (Table 1). The results were compared with safety limits for intrabeam viewing of laser radiation. Furthermore, the calculated mean irradiance at the corneal surface was compared with the irradiance measured with a calibrated photodiode (Silicon Sensors GmbH, Berlin, Type Si-PIN-SSO-PD-100-6). For all types the ratio between the calculated light energy entering the eye and the safety limits is equal to or less than 0.2, and the measured and calculated irradiances at the cornea differ by at most a factor of 2 (Table 1). The latter differences can be attributed to the large tolerances in emitted light power of the LED’s (factor of 2 or greater).

D. Details of the Electronic Equipment
The dark noise at the output of the voltage-to-current converter (built into the PMT unit) is 90 mV peak to peak, the signal of a healthy cornea is 210 mV, and the corresponding signal noise is 240 mV peak to peak (bandwidth, 20 MHz). The PMT signal is sent to a compact electronic unit for elaboration and display. The unit, a block diagram of which is shown in Fig. 4, is based on a single-chip microcontroller (Motorola Model 68HC11) and on a specifically designed elaboration board. In addition, the unit contains the power supply for all the LED's operating in the optical head and for the IR transmitter–receiver combination.

The electronic elaboration board, fully controlled by the microcontroller, performs single-channel gated integration of the PMT signal within a programmable time interval, using a low-noise operational amplifier (Burr-Brown Model OP-27). A second amplifier and shaper unit is used to handle the IR signal and to drive the earphone set.

The microprocessor unit is interfaced with the elaboration unit through its built-in analog-to-digital (A/D) converter and digital input–output (I/O) ports. Additional I/O and serial ports are used to control the LC display that is used to display the messages and the results of the measurement to the operator. The microprocessor was programmed directly in assembler language, given the inherent simplicity of the
The measurement steps for acquisition of the corneal fluorescence were conveniently designed to optimize the signal-to-noise ratio of the measurement and to minimize the unwanted contribution of the crystalline lens to the signal. We describe the measurement sequence with the aid of the temporal diagram in Fig. 5.

At power on, all light sources are switched on and off alternately so they can be checked qualitatively. Then the measurement is started. In this phase, both the set of green LED's and the red LED are on. The red LED, which in this phase of the measurement is used only for aiming, is operated at low current. The operator can position the optical head at the correct place with respect to the target eye by looking at the viewer of the camera and moving the camera until buzzing in the earphone stops and the cornea is properly positioned.

Pressing the camera shutter (with the camera in the B position) initiates the following sequence of events (time C in Fig. 5): First the green LED's and the red LED are turned off. Then, after a convenient delay, the blue LED's are turned on for a preset time interval and the elaboration board integrates the fluorescence signal over this interval. After this time interval the output of the gated integrator is transferred to a sample-and-hold circuit and converted by the A/D converter. The gated integrator is then zeroed by a controllable field-effect transistor switch. Following this procedure, the blue LED's are turned off and the acquisition of the background signal is performed in exactly the same way. The background signal is then subtracted from the fluorescence signal. When the signal-to-background ratio subtraction is finished, the red diode is turned on at full current to prevent the pupil from dilating. After the red LED is turned off, the sequence starts again. The signal-to-background ratio acquisition is repeated 16 times, and the result is averaged. These repetitive sequences are required because the pupil starts to enlarge \( \sim 200 \text{ ms} \) after the front illumination has been turned off. In the virtual instrument turn-on duration, the delay, and the number of measurements are selectable. In the stand-alone system a preoptimized set of parameters is available.

At the end of the measurement the following data are available: average fluorescence value and standard deviation over the single measurements, together with the maximum value of the signal and of the background, to assist the operator in setting the appropriate level of remaining ambient illumination to prevent saturation of the photomultiplier. Note that the apparatus is used normally in near dark; an alarm is set in case of A/D saturation. The corrected corneal autofluorescence value is presented on a digital display (with the virtual instrument, all the data are saved on file together with the parameter settings for further elaboration and presentation).

### 3. System Characterization

#### A. Axial Sensitivity of the IR Transmitter–Receiver Combination

We tested the axial sensitivity of the IR transmitter–receiver combination to check the accuracy in positioning of the target before measurement. We mounted a standard 1-cm glass cuvette onto a translocation unit positioned between the two prisms, connected the output of the IR amplifier to an oscilloscope, and monitored the output voltage, varying the position of the cuvette relative to the optical head. The results are shown in Fig. 6. The 10–90% range of positioning the camera in forward–backward directions was no larger than 0.4 mm. Incidentally, after brief training it is easy for the operator to distinguish the position where the acoustic signal is 10% of the maximum and to choose this as the optimum value. This range implies that the camera can be positioned at a fixed distance from the cornea with an accuracy of \( \sim 0.1 \text{ mm} \), which can be stated as “in a reproducible way.”

The sample fluorescence volume contains a fixed part of the cornea, including the epithelium and the endothelium because the cornea is quite transpar-
Moving the cornea forward or backward induces almost no change in the sample fluorescence volume, and the excitation light in that range is homogeneous within 30% so these kinds of movement (e.g., as a result of respiration) have almost no effect on the fluorescence signal registered. Slight movement of the eye to the left or to the right is compensated for by the balanced left-right excitation illumination. Note also that fluorescence measurements last 10 s and thus result in an averaging out of the effects of small movements.

B. Linearity and Sensitivity

The linearity and sensitivity of the corneal fluorometer were measured with a set of square quartz cuvettes of 10-mm width filled with solutions of NaFl (with excitation and emission maxima $\lambda_{\text{exc}} = 490$ and $\lambda_{\text{em}} = 520$ nm, respectively) in a buffer medium at pH 7.4, with concentrations ranging from 0 to 200 ng/mL as measured by a spectrophotometer. For every concentration, 30 repeated measurements were performed in sequence. Figure 7 shows a plot of average values (solid curve, left scale) and standard deviation (dashed curve, right scale) for all the concentrations tested. The standard deviation of these NaFl measurements can be seen to be less than 1% in the range of concentrations mentioned. The correlation coefficient is $r = 0.999$, $P < 0.001$, and the coefficient of relative deviation is 3.3%.

We determined the spatial fluorescence sensitivity by measuring the fluorescence signal at the output of the PMT of a small sphere of (polyester-embedded NaFl), which was moved in three directions.
resulting sensitivity plots are presented in Fig. 8. Figure 8(a) demonstrates high sensitivity in the left–right directions and a minimal sensitivity in the central position as a result of the physical obstruction by the red LED. The difference between the left and right intensity peaks was proved to originate from a difference in blue light power. We eliminated the difference in a later version of the instrument by adjusting the LED currents. Note also the low sensitivity in the central upward direction [Fig. 8(b)] and along the optical axis of the instrument, at the normal position of the human lens and without screening by a constricted iris [Fig. 8(c)].

4. Experimental Results

A. Repeatability

The instrument was tested for repeatability. One of the cuvettes of NaFl was used for the test. The cuvette was positioned at the optimum position as detected with the earphones, and a series of subsequent measurements was performed over a time of 2 h. The standard deviation of the readout values was ~1% of the average value and over 30 measurements. To test the in vivo repeatability we measured the corneal fluorescence of one of the authors (Docchio). The system was realigned before each measurement, and several untrained volunteers performed the measurements on different days. After a short period of training, the measurement variations were found to be less than 3%. Similar results were obtained with measurements made by another volunteer. This result also demonstrates the ease of operation of the instrument by poorly trained, nonclinician operators.

B. Diabetic versus Healthy Subjects and Comparison with an Existing Instrument

The values of corneal fluorescence of healthy volunteers and diabetic patients obtained with the fluorometer were compared with those obtained with a commercial scanning fluorophotometer ($\lambda_{exc} = 490 \text{ nm, } \lambda_{em} = 520 \text{ nm; Fluorotron Master, OcuMetrics; Fig. 9}$). That instrument scans the fluorescence stepwise along the optical axis of the eye. The autofluorescence at the position of the cornea is usually increased by the amount of residual autofluorescence of the lens as the result of the limited spatial resolution of the scanning fluorophotometer (~0.5 mm). We corrected for this lens tailing by subtracting the residual lens curve from the corneal scan. The residual lens curve was obtained by exponential interpolation of the lens curve at the position of the cornea.$^{15}$

The healthy volunteers were obtained from among our colleagues at the Leiden University Medical Center and their relatives. The patients were obtained through the Ophthalmology Outpatient Department. Patients had type-II diabetes mellitus with different levels of retinopathy graded by the Airlie House classification$^{8,23}$ [three patients with no or negligible retinopathy, five with background retinopathy, and two with (pre)proliferative retinopathy, corresponding to grades 1, 2 and 3 combined, and 4, respectively]. Grades 2 and 3 were combined because no significant differences in corneal autofluorescence had been found among patients in these groups in previous investigations.$^{8,14}$ The research followed the tenets of the Declaration of Helsinki, and informed consent was obtained after the nature and possible consequences of the study were fully explained. The ages of patients as well as of the healthy control subjects ranged from 20 to 50 years.

Figure 9 shows these preliminary results of the measurements on the diabetic and healthy subjects. In the abscissa the reading obtained with the scanning fluorophotometer is reported [expressed in nanograms per equivalent milliliter of NaFl (ng/mL)], whereas in the ordinate the reading from the portable fluorometer (in arbitrary units) is shown. Open symbols represent readings from healthy subjects; filled symbols, those from diabetic patients. The correlation coefficient amounted to 0.7 ($P = 0.002$), and the coefficient of relative deviation was 34%.

C. Calibration of the Instrument

An important measurement that was not performed at this stage of instrumental development is the calibration of the instrument. Although the solid-state LED's that were used as light sources in the instrument and the detection device do not degrade rapidly, identical instruments should give the same results in the same patients. Therefore we envisage the use of a fluorescing device in the shape of a cornea, e.g., a contact lens made of polyester-embedded NaFl, a material with fluorescence characteristics suited for comparison with those of the cornea.$^{22}$ This device should be positioned in a standardized way in front of the instrument just as the cornea is. The sensitivity of the instrument can then be adjusted in such a way that the fluorescence signal is equal to a preset value.
5. Conclusions
A prototype of a new corneal fluorometer has been presented that we believe can be successfully used in the routine screening of population at risk for diabetic retinopathy. The apparatus has proved to be easy to use, automatic, flexible, sensitive, linear, and repeatable. Clinical screening of selected diabetic populations with or without retinopathy is necessary to validate the system. We envisage that this screening can be carried out successfully by taking advantage of the existing European Network of clinics participating to the Concerted Action on “Ocular Fluorometry: Standardisation and Instrumentation Development” of the 4th European Community Medical and Health Research Programme (MR 4*/0134/P). The screening, if positive, will decide the effectiveness of the system as a low-cost, compact, and inexpensive system with a valid diagnostic potential for populations at risk for diabetic retinopathy.

The present research was conceived within the framework of the concerted Action on “Ocular Fluorometry: Standardisation and Instrumentation Development” of the 4th European Community Medical and Health Research Programme. The authors greatly acknowledge the invaluable contributions of C. Alkemade, E. P. M. Boets, E. Dekker, J. L. van Delft, J. Fakkel, and T. R. Stolwijk of the Leiden University Medical Center, Leiden, The Netherlands, and of G. Castioni, G. Coffetti, A. Flammini, E. Gelmini, U. Minoni, and L. Rovati of the University of Brescia, Brescia, Italy, during various phases of the development.

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