

## Simultaneous Noninvasive Clinical Measurement of Lens Autofluorescence and Rayleigh Scattering Using a Fluorescence Biomicroscope

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### Abstract

#### Background:

Lens autofluorescence increases with the age of the subject, and the fluorophores responsible are associated with cataract, retinopathy, and other complications of diabetes. We built a scanning confocal lens fluorescence biomicroscope suitable for routine clinical measurement of lens autofluorescence and light scattering and report data from 127 healthy subjects.

#### Method:

The fluorescence biomicroscope focuses a beam of light from a blue light-emitting diode on the lens and measures fluorescent green light and blue scattered light using a sensitive silicon photomultiplier. The system includes a target fixation light and a video camera for alignment and automatic pupil tracking. Under software control, a volume of measurement is scanned from behind the posterior lens capsule, through the lens to the aqueous humor, and then back again. Software computes the average ratio of lens autofluorescence to scattered light in the central portion of the lens. Self-reported healthy nondiabetic subjects were examined by an optometrist; if their eyes were healthy and without significant cataract, they were entered into the study.

#### Results:

Valid lens autofluorescence data were collected from 127 subjects between 21 and 70 years of age. A linear model for lens autofluorescence intensity with age was highly statistically significant, and the improvement in fit for higher-order polynomial models was not statistically significant. The ratio of lens autofluorescence to light scatter was also calculated; regression analysis showed significant curvature for the relationship of the fluorescence ratio to age, so a nonlinear model was used to estimate the mean ratio of autofluorescence to scatter and its prediction intervals as a function of age.

*continued →*

**NOTE:** The ClearPath DS-120 Lens Fluorescence Biomicroscope is indicated for use to detect autofluorescence of the crystalline lens. The clinical uses described in this information have not been approved or cleared by the FDA

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**Abbreviations:** (AGE) advanced glycation end product, (IR) infrared, (DFA) 1-deoxyfructosyl glycation adduct, (LED) light-emitting diode, (UV) ultraviolet

**Keywords:** advanced glycation end products, biomicroscope, diabetes mellitus, fluorescence ratio, lens autofluorescence

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**Abstract cont.**

**Conclusions:**

Our observation of a strongly significant linear regression of fluorescence intensity with age of the subjects agrees with the results from previous studies, as does a nonlinear model for the fluorescence ratio. The fluorescence biomicroscope enables the clinician to identify patients with fluorescence ratio significantly higher than expected for their age.

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## Introduction

The study of the autofluorescence of the human crystalline lens has a long history, beginning with reports from the 19th century.<sup>1,2</sup> Early clinical studies established that lens autofluorescence could be measured noninvasively and that fluorescence intensity increases with the age of the subject.<sup>3,4</sup>

That a noninvasive measurement of lens autofluorescence may have clinical utility is suggested by its physiological basis, which is the accumulation of “advanced glycation end products” (AGEs), a heterogeneous family of yellow-brown and fluorescent proteins that have been modified by glycation (the nonenzymatic reaction of reducing sugars with the free amino groups of proteins and subsequent irreversible reactions).<sup>5</sup> Although most proteins in living systems turn over with sufficient rapidity to avoid significant accumulation of AGEs, some, such as lens crystallins, nerve myelin, and skin collagen, are long lived, and AGEs accumulate in these proteins over a person’s lifetime.<sup>5,6</sup> The accumulation of AGEs is believed to contribute to the gradual decline in tissue and organ function that is observed within the aging body.<sup>7</sup> Advanced glycation end products have been implicated in diseases of aging, including cardiovascular diseases.<sup>8-13</sup>

Because glycation is initiated by free reducing sugars, diabetes subjects have an accelerated accumulation of AGEs.<sup>14-22</sup> Thus lens autofluorescence, as a noninvasive measure of AGE accumulation, can be regarded as a measure of cumulative tissue damage due to elevated sugar in plasma and interstitial fluids.

The clinical application of lens autofluorescence has been limited by the unavailability of an instrument suitable for routine clinical use. We have developed, and are seeking U.S. marketing clearance for, a scanning confocal

lens fluorescence biomicroscope with blue light-emitting diode (LED) excitation that rapidly measures green fluorescence within a volume of measurement that is scanned through the lens. Novel features include the ability to simultaneously measure blue Rayleigh scattered light and an infrared (IR) video system that detects and automatically tracks the position of the pupil so that measurements are fully automated. It is not necessary to dilate the pupil. By computing the ratio of fluorescence to scatter, most potential sources of measurement variation are eliminated, and an easily interpreted index is reported to the clinician.

To establish a baseline of normal eyes, we have used this instrument to measure the fluorescence intensity and fluorescence ratio of 127 self-reported healthy subjects from 21 to 70 years of age with healthy eyes, as verified by optometric examination.

## Methodology

### *Biomicroscope*

The ClearPath DS-120® lens fluorescence biomicroscope optical system (Freedom Meditech, San Diego, CA) consists of a blue (465 nm) LED excitation light with a 430–470 nm band-pass filter, source and collection focusing optics, motor-driven filter wheel with 25% neutral density (scatter), and long-pass (fluorescent emission 500–1650 nm) filters and a silicon photomultiplier light detector (**Figure 1**). In addition, there is a red blinking LED target fixation light positioned within red blinking concentric rings to aid the patient in self-alignment, three IR LED lights to illuminate the eye, and a video camera. A fluorescence reference target is positioned in the optical path during the self-test procedure at startup. Stepper motors control the optics window horizontal motion, chin rest motion,

filter wheel rotation, and optical focus point  $x$ ,  $y$ ,  $z$  positioning.

### Startup Procedure

The system performs a self-test when the fluorescence biomicroscope software starts up. The self-test procedure verifies computer communication with the optical system; resets and zeros the  $x$ ,  $y$ ,  $z$  positions of the focal point with respect to its switch limits; and starts the filter wheel. The self-test also verifies that the device is operating within normal limits by measuring the fluorescence reference target.

### Operation

Only the patient's chin and forehead contact the ClearPath DS-120 lens fluorescence biomicroscope. The patient is positioned with the forehead centered on the headrest. The eye is illuminated by three near-IR 880 nm LED lights and is observed by an IR-sensitive charge-coupled device video camera. An image of the eye is displayed on the computer screen to assist the operator in the alignment of the patient.

The headrest is adjusted manually to bring the corneal plane of the test eye close to the optics window so that the eye is in focus. The patient is instructed to look at the red blinking LED fixation light that is surrounded by red blinking concentric rings. The operator adjusts the optics window and chin rest by clicking arrow icons on the computer screen.

The software includes an automatic tracking program for positioning the pupil. The system automatically aligns its optical axis before a measurement is taken.

The patient is instructed to close and open the eye (to wet the cornea with a tear film to reduce blinking) and the operator clicks on the start icon to begin the scan.

The blue LED light source is focused to achieve a converging excitation beam of blue light that is initially positioned just behind the posterior lens capsule. The collection optics are confocally aligned within a 1 mm diameter and 3 mm long volume of measurement that is scanned through the lens in 0.31 mm steps (**Figure 2**). In the eye, this blue light is scattered by elastic (Rayleigh scattering) and inelastic (fluorescent) interactions with lens proteins.

A filter rejects red and IR light from the positioning lights. A rotating filter wheel chops the beam into blue/green (primarily Rayleigh scattered) and green (fluorescent)

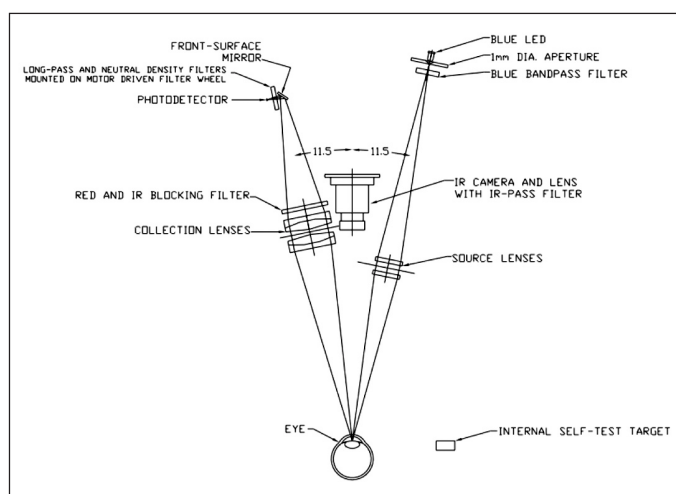


Figure 1. Optical system. Dia, diameter.

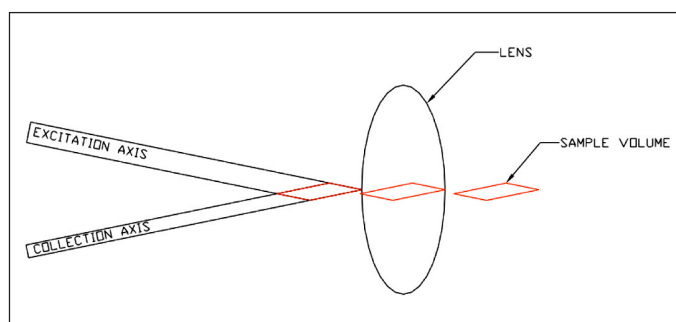


Figure 2. Diagram of volume of measurement.

segments. The scattered and fluorescent light is focused on a highly sensitive silicon photomultiplier, and the signals are sent to the analog-to-digital converter on the optics control board and then to the computer.

Under software control, the volume of measurement at the focal point of the light source and detector is scanned from just behind the posterior lens capsule, through the lens, through the anterior lens capsule to the aqueous humor, and then back again. Computer software records both scattered and fluorescent light during the forward and reverse scan and constructs a graph of each that is displayed on the computer monitor. Software detects the front and back surfaces of the lens capsule on the graph, estimates the apparent thickness of the lens, and computes the average of the ratio of lens autofluorescence to scattered light in the central portion of the lens. The software checks that the apparent lens thickness is within a physiological range. Software also detects anomalies in the scan, such as eye blinks, which could cause an inaccurate measurement, or excessive difference between the two scans.

For valid scans, the fluorescence ratio is reported; otherwise, for anomalous scans (too much difference in forward and reverse scans, anterior and posterior edges outside of physiological range, pupil too small, signal out of linear range, or fluorescence ratio outside of physiological range), an error code is reported on the computer monitor, and the fluorescence ratio is not reported. In the case of an error code, the clinician can rescan the eye.

If the scan is valid, software produces a report that is displayed on the screen and that can be printed for the patient and/or for the patient's file. The scan data are also automatically saved on the computer's hard disk.

### *Human Participant Selection Criteria*

Each participant signed an informed consent document, filled out a health questionnaire, and underwent an eye examination (including a slit lamp examination) by an optometrist before being tested with the study device. The following criteria were used to select patients:

#### Inclusion Criteria

- Subject must be aged between 21 and 70 years,
- Subject must be able to read or understand English and give informed consent,
- Subject must complete the health questionnaire, and
- Subject must have healthy eyes, as determined by the optometrist.

#### Exclusion Criteria

- Subject is not deemed healthy based on the comprehensive eye exam;
- Subject has type 1 or type 2 diabetes, as reported on the health questionnaire;
- Subject has had the crystalline lens removed from the test eye or has had the crystalline lens removed and replaced with an intraocular lens implant in the test eye;
- Subject has had a fluorescence angiogram within the past 6 months;
- Subject has undergone a treatment using photodynamic drugs within the past year;

- Subject has been clinically diagnosed with cataracts in the test eye, with any sign of opacification;
- Subject has ocular surface (dry eye) disease;
- Subject is unable to cooperate with or understand clinical instructions; or
- Subject is unable to complete test sequence.

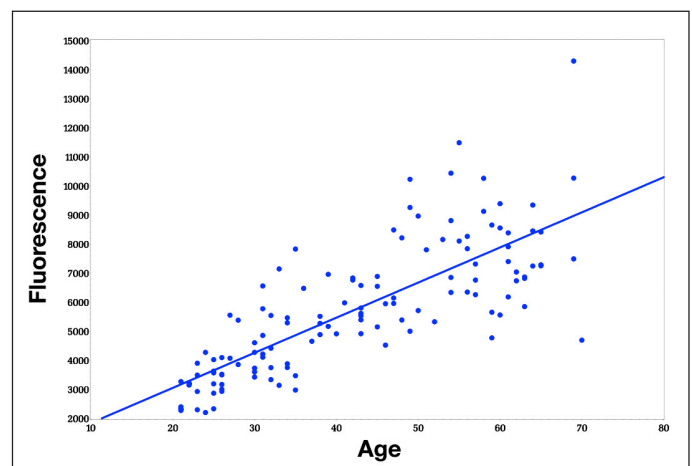
Prior to performing the study, each operator went through a familiarization period. Familiarization included reading and understanding the procedure for the instrument and successfully performing a scan.

### *Fluorescence Intensity Measurement*

Subjects were scanned up to a maximum of eight scans; however, testing was terminated after five successful scans on the subject. A successful scan is one for which the instrument reports a fluorescence ratio instead of an error code. Of the 128 recruited into the study, 1 subject had no successful scans because of droopy eyelids and excessive blinking. There were a total of 628 successful scans among the 127 subjects that had at least one successful scan (mean, 4.9 successful scans/subject).

### *Data Collection*

In addition to reporting the fluorescence-intensity-to-scatter ratio to the clinician on the screen (as previously described), the fluorescence biomicroscope records fluorescence intensity and other scan details in a file on the computer's hard disk. The fluorescence intensity data shown in **Figure 3** was obtained from this file.



**Figure 3.** Mean fluorescence (arbitrary units) from healthy eyes of 127 healthy subjects versus age (years) from clinical study with regression line (fluorescence = 368 + 138 × age).  $R^2 = 0.72$ .

Demographic data were analyzed with the Frequencies procedure for SPSS version 19 software for Mac OSX (version 10.6) for descriptive data and the Means procedure for means, medians, and ranges. Fluorescence ratios were analyzed with SAS 9.1 for Windows with procedure PROC GLMMIX.

## Results

### Demographics

The study enrolled 128 men and women. One subject, a 41-year-old Asian male, had no valid scans and was removed from the data set. Thus there were 127 valid subjects in the data set.

Demographics of the patient population are shown in **Tables 1–4**. A small majority of the subjects were women. Ages ranged from 21 to 70 years, with a median age of 42 years.

### Subject Health Characteristics

Subjects who were enrolled were self-reported as healthy. Health parameters that subjects reported included cholesterol, hypertension, kidney disease, diabetic first-degree relative, and smoking (**Table 5**).

### Regression of Fluorescence Intensity versus Age

We performed a linear regression of mean subject fluorescence intensity versus age in years. Since Levene’s test showed a highly significant heterogeneity of the variances of the deviations from the mean of replicate observations for each subject, the subject means were weighted using the inverse of the subject-specific variance. The linear model was highly significant, with  $p \ll .001$  (**Table 6**). The improvement in fit for higher-order polynomial models was not statistically significant. **Figure 3** shows the subject mean fluorescence intensities and the linear regression model versus age.

### Model of Fluorescence Ratio versus Subject Age

The instrument achieves additional reliability by use of a ratio method that removes many of the potential sources of variation that apply to the measure of absolute fluorescence intensity. Thus, in addition to measuring green fluorescence, the instrument also measures blue scattered light. We used the scattering measurement to calculate the ratio of fluorescence intensity to scatter in order to correct the fluorescence for attenuation effects.

Replicate measurements of individual subjects showed a 5.5% coefficient of variability. Polynomial regression of

Gender	Frequency	Percentage
Female	71	55.9
Male	56	44.1
Total	127	100.0

Age range	Frequency	Percentage
20–29	30	23.6
30–39	30	23.6
40–49	23	18.1
50–59	22	17.3
60–69	21	16.5
70	1	0.8
Total	127	100.0
Mean age	42.4	
Median age	42.0	

	Frequency	Percentage
Asian/Pacific Islander	19	15.0
Black	4	3.1
Caucasian	86	67.7
Hispanic	10	7.9
Multi-race or other	8	6.3
Total	127	100.0

Gender	Statistic	Height (inches)	Weight (pounds)	Body mass index
Female	Mean	63	133	22.7
	Median	64	130	21.6
	Minimum	49	95	18.3
	Maximum	72	200	33.8
Male	Mean	69	184	25.9
	Median	69	176	25.8
	Minimum	61	127	19.5
	Maximum	77	320	42.2

Table 5. Self-Reported Health		
	Frequency	Percentage
High cholesterol	17	13.4
Hypertension	9	7.1
Kidney disease	0	0.0
Diabetic first-degree relative	33	26.0
Smoking	42	33.1
Total	127	100.0

the fluorescence ratio against age showed significance for quadratic term; inclusion of gender, ethnicity, and self-reported health variables did not significantly improve the fit of the model to the data. Because the quadratic function shows a nonphysiological negative slope at high age, we used a nonlinear model to compute the mean predicted ratio and 80% and 95% prediction intervals (Figure 4):

$$y = \kappa / (1 + \exp(-\beta * (x - \mu)))$$

where  $y$  is the fluorescence ratio,  $x$  is the subject age,  $\kappa$  is the level of asymptote,  $\beta$  is the rate of rise, and  $\mu$  is the location of the inflection point. The parameters  $\kappa$ ,  $\beta$ , and  $\mu$  are estimated using nonlinear least squares regression, using only a single measurement for each subject in order to obtain the prediction interval for a single observation. The model was highly significant ( $p < .0001$ ), with estimates of  $\kappa = 0.22$ ,  $\beta = 0.15$ , and  $\mu = 20.4$ .

## Discussion

Our observation of a strongly significant linear regression of fluorescence intensity with age of the subjects agrees with the results with previous studies (except for differences of scale).<sup>23-25</sup> Similar relationships have been reported for skin autofluorescence.<sup>26-28</sup>



Figure 4. Observed fluorescence ratios from healthy eyes of 127 healthy subjects from clinical study with regression line (green) and upper one-sided 80% and 95% prediction intervals.

## Significance of Lens Autofluorescence

Kurzel and coauthors<sup>29</sup> identified fluorophores with five emission maxima in the ultraviolet (UV) and visible fluorescence spectra in the intact human lens. The UV bands include the amino acid tryptophan, a natural fluorophore in proteins, as well as other fluorophores that are responsible for lens autofluorescence that increases with age. A blue/green (450 nm excitation/550 nm emission) fluorophore, corresponding to the excitation and emission bands used in this study, is substantially increased in brunescens lenses.<sup>29</sup>

The fluorophores responsible for age-dependent lens fluorescence have been chemically identified as AGEs that are formed by a process of glycation.<sup>6,30-32</sup> Abiko and coauthors<sup>33</sup> measured lens autofluorescence in Chinese hamsters *in vivo*, removed the eyes, and analytically measured AGEs in lens extracts; lens autofluorescence was correlated with AGEs as measured by enzyme-linked immunosorbent assay. Other studies that identify AGEs

Table 6. Analysis of Variance for Linear Regression of Fluorescence Intensity (Arbitrary Units) versus Age (Years) Shown in Figure 3						
	Model	Sum of squares	df	Mean square	F	Significance
1	Regression	141,872	1	141,872	325	0.000
	Residual	54,457	125	435		
	Total	196,329	126			

as the fluorophores responsible for lens autofluorescence include Kamei and Kato,<sup>32</sup> who correlated the analytically determined 1-deoxyfructosyl glycation adduct (DFA) with the fluorescence of normal and colored human lenses of different ages. Both DFA and lens autofluorescence were shown to be reliable indicators of the extent of glycation, and both increased significantly with chronological age and lens brunescence. Das and coauthors<sup>34</sup> used immunochemical assays to quantitate AGEs, which were shown to be correlated with lens fluorescence.

Advanced glycation end products, measured chemically in lens extracts, are also correlated with age and are elevated in cataractous and brunescence lenses.<sup>21,32,34–43</sup> Thus increased lens autofluorescence can be a biomarker indicative of more extensive underlying damage to the proteins due to glycation.<sup>5,32</sup>

### Clinical Studies of Lens Autofluorescence

Consistent with the identification of lens autofluorescence with the accumulation of AGEs due to glycation reactions, noninvasive clinical studies of blue/green lens autofluorescence have shown that, in addition to the increase of lens autofluorescence with age,<sup>25,44</sup> lens autofluorescence is increased in patients with diabetes mellitus.<sup>23,45–49</sup> Furthermore, within the diabetes patient population, lens fluorescence is also correlated with the development of the diabetes-related pathologies of cataract and retinopathy<sup>50–52</sup> and with systemic pathologies.<sup>53</sup>

### Fluorescence Ratio versus Age

Yu and coauthors<sup>54</sup> suggested that the ratio of fluorescence intensity to scattering would help compensate fluorescence measurements for attenuation effects; their clinical data indicated that the fluorescence ratio could provide better discrimination between individuals with and without diabetes than fluorescence intensity. Our data showing a nonlinear relationship of fluorescence ratio to age in healthy subjects are consistent with the data presented by Yu and coauthors.<sup>54</sup> Our data are based on only healthy subjects, so we carried out a nonlinear regression to generate predicted fluorescence ratios versus age as well as 80% and 95% prediction intervals. There were 9 subjects above the 95% confidence interval, which is greater than the 6 expected for the 127 patient sample, suggesting the possibility that some of the subjects who self-reported as healthy may have developed diabetes. However, the study is limited in the numbers of subjects in the higher age groups and by the lack of confirmative laboratory data. In the future, we plan to carry out

clinical trials on both healthy subjects and subjects who have type 2 diabetes with sufficient power to determine the ability of the fluorescence ratio to screen for risk for undiagnosed diabetes.

## Conclusion

The fluorescence biomicroscope enables the clinician to measure lens autofluorescence in routine eye examinations. Patients with lens autofluorescence significantly higher than expected for their ages may have an accelerated accumulation of AGEs and increased risk for the pathologies associated with diabetes and AGEs.

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### Disclosures:

Frederick Cahn is a consultant to Freedom Meditech. John Burd and Keith Ignatz are employees of Freedom Meditech.

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