A Scanning Ocular Spectrofluorophotometer

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We describe an instrument called a scanning ocular spectrofluorophotometer (SOSF) that measures fluorescence in a two-dimensional cross-section through the anterior chamber and cornea and provides the ability to change excitation and emission wavelengths rapidly. The output of a xenon arc lamp is filtered by a diffraction grating monochromator which has a bandpass of 4 nm and a range of 400 to 800 nm. Light emitted from the fluorophore is filtered by a variable wavelength interference filter which has a bandpass of approximately 11 nm and a range of 400 to 700 nm. To demonstrate the versatility of the instrument, we measured the spectra of fluorescein, fluorescein glucuronide and rhodamine B in the anterior chambers and corneas of pigmented rabbits after topical administration. We also measured simultaneously and independently the redistribution and disappearance of a mixture of fluorescein-labeled dextran and rhodamine B after intracameral injection. Rhodamine B was very rapidly absorbed by the cornea and lens while fluorescein-dextran was not measurable in the cornea before 4 hr. The SOSF provides a means of carrying out spectrofluorophotometry in the living eye and carrying out kinetic experiments which would otherwise be awkward or impossible. Invest Ophthalmol Vis Sci 29:1285-1293, 1988

Considerable information has been gained from studying changes in the concentration of fluorescent dyes in the eye as a function of time. Additional information is available if other properties of fluorescence are measured in addition to intensity at a single wavelength. For example, by measuring fluorescence at two excitation wavelengths, one can separate concentrations of two fluorophores in a mixture, such as fluorescein and its metabolite fluorescein glucuronide. Changes in pH have recently been measured in the aqueous humor and cornea by measuring fluorescence at two separate excitation wavelengths. There is the potential that some components of aqueous humor such as ascorbate can be measured by observing the conversion of fluorescent reagents in vivo. Thus, measurement of fluorescence at more than one wavelength can provide information that measurement of total fluorescence cannot. Excitation and emission spectra of fluorophores in solution can easily be measured in a cuvette with any of a number of commercially available spectrophotometers. Measurements of spectral properties of fluorophores in the eye require specialized equipment. In this paper, we describe a spectrophotometer designed to make these measurements in the living eye. The focal point of the instrument scans across its target while making measurements. Hence, we refer to the instrument as a scanning ocular spectrofluorophotometer (SOSF).

Materials and Methods

Rationale and Goals

Our goal was to design an instrument that would allow noninvasive measurement of fluorescence in the anterior chambers and corneas of human or animal subjects without discomfort to the subject. Four modes of operation were defined. First, the instrument must measure fluorescence in a two-dimensional cross-section through the anterior segment (spatial scanning) at any fixed excitation and emission wavelength from 400 nm to 700 nm. This feature would allow measurement of a large number of potentially useful fluorophores. Second, it must be capable of toggling between two sets of excitation and emission wavelengths on alternate spatial scans. This feature would allow near-simultaneous measurement at different wavelengths to differentiate mixtures of fluorophores. Third, it must be capable of stepping through a range of excitation wavelengths to measure the excitation spectrum of a fluorophore. Finally, it must be capable of stepping through a range of emission wavelengths to measure an emission spectrum.
Fig. 1. Scan assembly. The measurement window is defined by the optics contained in the scan assembly. Light from the fiber optic guide is directed by mirror M1 and focused on the anterior segment by lens L1. Fluorescent light is directed toward the detection assembly by lens L2. As the scan assembly moves in an anterior-posterior direction, the measurement window scans through the cornea and anterior chamber, and anterior crystalline lens.

The following paragraphs describe the instrument that was designed and constructed to meet these goals.

Description of Instrument

The excitation light source is a 75 W xenon short arc lamp. The image of the arc is focused onto the entrance slit of a 0.125 m diffraction grating monochromator (Photochemical Research Associates, London, Ontario, Canada, model B102). The image of the output slit is focused onto a 0.6 mm diameter, silica fiber optic guide (single fiber) which conducts light into the instrument. The bandpass of the monochromator is approximately 4 nm and can be made narrower at the expense of intensity of the excitation light at the eye. The total power of the resulting beam which irradiates the eye is approximately 3 μW at 490 nm. The spectroscopic range of this excitation system is 400 nm to 800 nm.

The fiber optic guide is routed into the scan assembly which contains optical components to focus the excitation light on the eye and to collect emitted light from the eye (Fig. 1). The light emitted from the fiber is reflected from a mirror (M1) and brought to focus in the eye with a lens (L1). The end of the fiber is masked so that its image, which is in focus in the anterior segment, approximates a rectangle 0.3 mm high by 0.6 mm wide. Fluorescent light is collected by an objective lens (L2) in the scan assembly and relayed to a second lens (L3) which focuses it onto a rectangular aperture 0.6 mm wide (Fig. 2). The angle between the excitation and emission axes is 55°. The working distance from L1 and L2 to the cornea is 120 mm.

Behind the rectangular fieldstop is a variable wavelength interference filter, a shutter and a photomultiplier tube. The interference filter has a bandpass of about 11 nm and a range of 400 nm to 700 nm (Optical Coating Laboratories, Inc., Santa Rosa, CA, model VC 180-017). The desired wavelength is selected by rotating the circular filter with a stepper motor that is under computer control. The photon-counting photomultiplier tube (Hamamatsu Corporation, Middlesex, NJ, R955P) is sensitive from less than 200 nm to 800 nm.

The measurement window is the intersection of the focused excitation path and the image of the measurement aperture projected back to the eye. The height of the measurement window is 0.3 mm, the width 0.6 mm and its anterior-posterior depth, approximately 0.7 mm. When measuring concentrations of a fluorophore in the cornea, the measurement window does not fit entirely within the anterior-posterior bounds of the cornea. The fluorescence signal is therefore underestimated and must be corrected. The correction factor is a function of cornea thickness and was determined by measuring fluorescence of a solution of fluorescein in a specially constructed chamber. The front surface of the chamber was a clear plano contact lens and the back surface was an opaque contact lens. The space between the front and rear lenses was filled with a solution of fluorescein and could be adjusted from 0.1 mm to 5 mm. The apparent fluorescence in the chamber at each of a series of thicknesses was measured. The ratio of fluorescence of the deep chamber (2 mm thick, a
point where changes in thicknesses made no difference in measured fluorescence) to apparent fluorescence in the chamber at the test thickness was determined and used to correct measurements of fluorescence from the cornea. Correction factors were determined for chamber thicknesses of 0.1 mm to 1.5 mm in steps of 0.1 mm. For a 0.5 mm thick cornea, the correction factor was 1.57.

The scan assembly is mounted on two parallel rods and bearings. A cam and a synchronous motor move this assembly to and fro in an anterior-posterior direction. The special cam, as it turns at a constant angular rate, displaces the lens assembly at a constant linear rate except during a brief period in which the direction of the lens assembly is reversed. The to and fro motion scans the measurement window along a line parallel to the optic axis of the eye. Since the light rays between L2 and L3 are parallel, the changing distance between them does not affect the focus or alignment. The cam rotates at 30 rpm; each unidirectional scan takes 1.0 second. The range of displacement of the scan assembly is 10 mm, although measurements are made only in the central 6 mm of this distance. Data are collected during each scan in 30 individual time periods, each lasting 17 msec. The corresponding volume of each measurement is called a "voxel."

The entire excitation and detection assembly is mounted on a pair of horizontal rods and bearings and four vertical rods and bearings. The assembly can be displaced laterally or vertically by a pair of constant speed motors while making repeated anterior-posterior scans. A two-dimensional image of fluorescence in a horizontal plane or vertical plane can be constructed from this two-dimensional scan.

The instrument is controlled by a Motorola 6809 microprocessor (Motorola, Inc., Austin, TX) that is driven by a program resident on an EPROM (Erasable Programmable Read Only Memory). The microcomputer performs real-time tasks including synchronizing the photon counting, storing data, changing wavelengths and opening and closing shutters. An IBM PC/AT serves as the master computer and provides a convenient means to set parameters, to receive, examine and display data, and to calibrate measurements. It also provides a system in which data can be saved and analyzed by applications programs.

Performance

The lower limit of detection of a fluorophore in the anterior chamber was defined as the equivalent concentration of the fluorophore that would produce a signal equal to two standard deviations above the noise signal from the anterior chamber with no fluorophore. This limit was dependent on the fluorophore and the wavelengths chosen for excitation and emission. For fluorescein, the excitation and emission wavelengths were 490 nm and 530 nm.

The short-term stability of the instrument was determined by measuring fluorescence from a 1.0 μM solution of fluorescein in a cuvette (λex = 490 nm, λem = 530 nm) at 30-second intervals. Before each ocular measurement, fluorescence was measured from a piece of fluorescent glass (Corning 3750, Corning, NY, measured at λex = 490 nm, λem = 520 nm). The fluorescence measured from the glass was used to correct ocular measurements for drift that may have occurred in the intensity of the light source or sensitivity of the photomultiplier tube.

The spectra measured with this instrument are dependent on several factors in addition to the spectral characteristics of the fluorescein material.26 The output of the xenon lamp, the throughput of the monochromator, lenses and fiber optic guide are wavelength-dependent. The transmittance of the variable wavelength interference filter and the response of the PMT also vary with wavelength. When spectra were graphed, a correction was made to compensate for the spectral sensitivity of the instrument. A correction factor was calculated at each wavelength from the ratio of fluorescence from fluorescein, pyranine, rhodamine B, and resazurin measured on a commercial spectrally corrected spectrofluorometer (SPF-500 C, SLM Instruments, Urbana, IL), to fluorescence measured on the SORS. Corrections were determined for both excitation and emission spectra.

The performance of the instrument was tested by measuring fluorescence from fluorescein in three human subjects. The subjects applied ophthalmic 2% fluorescein solution (Alcon Laboratories, Fort Worth, TX) to their eyes, one drop every 5 min for a total of five drops, starting at 3:00 AM on the day of the study. They then went back to sleep until their usual awakening time. Measurements were made every hour from 9:00 AM until 4:00 PM. Informed consent was obtained from each subject after the nature of the study had been explained and an eye examination had been performed.

Fluorescence from fluorescein, fluorescein glucuronide and rhodamine B was also measured from the anterior chambers and corneas of pigmented rabbits. An aqueous solution of 2% fluorescein (Alcon Laboratories), 2% fluorescein glucuronide (Chugai Pharmaceutical Co., Ltd., Tokyo, Japan), or 0.1% rhodamine B (Eastman Kodak Co., Rochester, NY) was applied topically to the cornea and the excess rinsed with saline solution after 5 min. Measurements were made every hour for the following 8 hr. During the measurements, animals were restrained in
a cloth bag as described previously. They were conscious and alert and did not show signs of discomfort or distress. Animals were handled and treated in accordance with the ARVO Resolution on the Use of Animals in Research.

The instrument was operated in four modes: (1) fixed excitation and emission wavelengths optimized for the specific dye; (2) measurement of excitation spectra; (3) measurement of emission spectra; and (4) simultaneous measurement of two fluorophores. In the fixed wavelength mode, the focal point was scanned in one of two ways: repeatedly along the central axis of the eye or axially and laterally to produce a two-dimensional measurement through a horizontal section of the anterior chamber and cornea. When spectra were measured, the measurement was made along the central axis only. Either the excitation or the emission wavelength was changed incrementally between consecutive scans until the spectrum was completed.

The intensity of fluorescence in the anterior chamber and cornea was obtained by employing an interactive program. Each axial scan was plotted on the computer monitor. The region of interest was selected by the user with a pair of cursors, and the mean intensity between the cursors was computed, labeled and stored. When intensities in the cornea and the anterior chamber at each wavelength in the sequence had been determined and saved, the spectrum (intensity vs. wavelength) was graphed.

Shifts in the wavelength of maximum excitation or emission were determined by fitting a spectrum of the fluorophore in a cuvette (reference spectrum) to the spectrum in the eye (test spectrum). The sums of the squares of differences between the reference spectrum and test spectrum at each wavelength were determined for a series of wavelength shifts of the reference spectrum. The wavelength shift of the test spectrum was assumed to be that which gave the closest match between the measured spectrum and shifted reference spectrum as determined by the least sum of squares.

A fourth mode of operation, simultaneous measurement of two fluorophores, was also tested. Pigmented rabbits were anesthetized with a mixture of ketamine and xylazine (45 mg/kg and 5 mg/kg i.m., respectively). The cornea was anesthetized with topical proparacaine. A needle-knife incision was made between the measured spectrum and shifted reference and rhodamine B (200 µM) into the anterior chamber with a 30 gauge needle through the incision. The injected volume was 10 µl. Measurements were made by repeated axial scans. Between groups of three axial scans, both monochromators were toggled between wavelengths optimal for fluorescein (λex = 490 nm, λem = 530 nm) and rhodamine B (λex = 555 nm, λem = 600 nm). These wavelengths were sufficiently separated that interference from rhodamine B when measuring fluorescein was approximately 1% of the total signal from rhodamine B (measured at λex = 555 nm and λem = 600 nm). Interference from fluorescein when measuring rhodamine B was less than 0.1% of the total signal from fluorescein (measured at λex = 490 nm and λem = 530 nm). A total of 12 scans was made, six at one combination of wavelengths and six at the other. Fluorescences at each combination of wavelengths in the anterior chamber and cornea were obtained and graphed as a function of time. No correction was made for changes in fluorescence efficiency in the cornea or lens due to binding.

Results

The lower limit of detection of fluorescein in the anterior chamber by the instrument was 0.3 ng/ml when measured at an excitation wavelength of 490 nm and emission wavelength of 530 nm. Measurements of a 1 µM solution of fluorescein in a cuvette (average of ten sample periods, 17 msec each), repeated every 30 seconds showed that measurements were repeatable to within 2% over 15 min.

Figure 3 shows two grey scale representations of fluorescence in the cornea and anterior chamber of a human subject after topical application of fluorescein. In the top figure, excitation was at 490 nm, near the excitation peak of fluorescein, and emission at 530 nm. The lower figure shows a grey scale representation of a scan made a short time later with an excitation wavelength of 450 nm. In Figure 4, fluorescence at both wavelengths is graphed as a function of anterior-posterior position for the central six scans of Figure 3. Fluorescence in the anterior chamber and cornea was approximately five times greater at 490 nm than it was at 450 nm. Notice that the anterior portion of the crystalline lens is more fluorescent at the short wavelength.

Fluorescence of fluorescein in the anterior segment at a series of excitation (fixed emission) and emission (fixed excitation) wavelengths is shown in Figure 5. Fluorescence in the cornea and anterior chamber increased as the excitation or emission wavelength moved toward the respective peaks for fluorescein. As noted above, fluorescence in the anterior lens did not follow this pattern, since little fluorescein entered the lens and the lens excites maximally at a shorter wavelength than fluorescein.

Excitation and emission spectra of fluorescein in the cornea and anterior chamber of a human subject are shown in Figure 6. Both spectra have been nor-


Anterior – posterior scan number

Fig. 3. Scans through the anterior segment at two wavelengths. Fluorescence in the cornea and anterior chamber of a human subject after topical fluorescein was more intense at an excitation wavelength of 490 nm (above) than at 450 nm (below). The horizontal axis represents distance across the eye, temporal to nasal (anterior-posterior scan number), and the vertical axis represents distance in an anterior-posterior direction (voxel number). The density of the shading is proportional to the log of the fluorescence.

malized to the maximum fluorescence and corrected for spectral characteristics of the instrument. In the cornea, the excitation spectrum was shifted by about 6 nm toward longer wavelengths as compared to spectra from the anterior chamber and a cuvette. This “red shift” is presumed to result from the fluorescein being bound by protein in the cornea.12 Emission spectra of fluorescein in the cornea were also red-shifted, although by only 2 nm to 3 nm. Excitation spectra of fluorescein in the anterior chamber were red-shifted by about 1 nm while emission spectra were red-shifted by less than 0.5 nm.

In the three rabbits, the red shift of the excitation spectrum was 9.5 nm to 12 nm in the cornea and 1 nm to 2 nm in the anterior chamber, somewhat greater than in humans. The emission spectra of fluorescein in the rabbits were shifted toward longer wavelengths by 6 to 10 nm in the cornea and 1 to 3 nm in the anterior chamber.

Spectra of rhodamine B in the rabbit eye are shown in Figure 7. Excitation spectra of rhodamine B in the cornea were red-shifted by 3 to 4 nm. In the anterior

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Fig. 4. Fluorescence intensity as a function of anterior-posterior distance. Fluorescence was approximately five times greater at 490 nm in the cornea and anterior chamber than at 450 nm. Fluorescence in the anterior pole of the lens was greater at 450 nm than 490 nm. The emission wavelength was 530 nm.

Fig. 5. Scans at different wavelengths. Excitation (above) and emission (below) spectra of a fluorophore in the anterior segment were measured by advancing the wavelength of the excitation or emission monochromators between consecutive A-P scans. A pair of cursors was used to select a region of interest and record fluorescence.
Unlike fluorescein, rhodamine B readily diffused into the anterior pole of the crystalline lens. Excitation spectra of rhodamine B in the lens were red shifted by about 4 nm while emission spectra were shifted toward shorter wavelengths ("blue-shifted") by 2.5 nm to 3.5 nm. The intensity of fluorescence from rhodamine B in the lens decreased much more slowly than from the anterior chamber or cornea. The half-life of rhodamine B in the lens was approximately 11 days while its half-life in the cornea and anterior chamber was similar to that of fluorescein, approximately 4 hr.

Fluorescence spectra from the cornea and anterior chamber after topical application of fluorescein glucuronide are shown in Figure 8. In contrast to fluorescein and rhodamine, the excitation and emission spectra of fluorescein glucuronide in the eye had a different shape than fluorescein glucuronide in a cuvette. Relative fluorescence intensities between 450 nm and 490 nm followed curves that were between those of pure fluorescein in the anterior chamber and fluorescein glucuronide in a cuvette. A similar change was found in the emission spectra. This spectral change is evidence that some of the fluorescein glucuronide was converted to fluorescein in the eye. The ratios of fluorescein glucuronide to fluorescein in the anterior chamber and cornea were calculated from the fluorescence intensities at 450 nm and 490 nm by using differential fluorophotometry. Four hours after application of pure fluorescein glucuronide, the average ratio of fluorescein glucuronide to fluorescein was 7.1 in the cornea and 14.8 in the anterior chamber.
Fig. 8. Excitation and emission spectra of fluorescein glucuronide. Normalized spectra in the anterior chamber and cornea were between spectra of fluorescein glucuronide in a cuvette and fluorescein alone in the anterior chamber. This indicates that some of the fluorescein glucuronide had been converted to fluorescein by the ocular tissues.

After injection of a mixture of fluorescein-labeled dextran and rhodamine B into the anterior chamber, the concentrations of the two dyes were measured in the anterior chamber and cornea (Fig. 9). Within 20 min, the two dyes were distributed quite differently. Rhodamine B, which is more lipid-soluble than fluorescein-dextran, rapidly penetrated the cornea and lens. In contrast, fluorescein-dextran initially showed no signs of entering either the cornea or lens. By 4 hr, some fluorescein-dextran appeared in the cornea as its concentration in the anterior chamber decreased. At 8 and 9.5 hr, fluorescein-dextran was prominent in the cornea. Both fluorophores continued to decrease, although rhodamine B decreased slightly faster than fluorescein-dextran (Fig. 10). The concentration of rhodamine B remained high in the lens throughout the experiment.

Discussion

Fluorescein has been used as a tracer to measure the flow of aqueous humor and the permeability of
barriers in the eye. Fluorophores with different physical properties might have advantages over fluorescein in certain experiments. For example, carboxyfluorescein is less lipid-soluble and is not converted to a fluorescent metabolite.13,14 Rhodamines are excited at longer wavelengths in a region of the spectrum where the natural fluorescence of the eye is lower. The use of monochrometers and a broad band light source and PMT in the SOSF and the fact that each monochromator is controlled by the computer eliminates all constraints on the selection of tracers as long as they are measurable within the visible spectrum. This feature makes the instrument particularly suitable for differential fluorophotometry for which rapid changes in wavelength are necessary to avoid artifacts due to the changing concentrations of the fluorophores in the living eye.

The ability to rapidly toggle between fixed pairs of wavelengths provides a means of studying physiological properties of the anterior segment by noninvasively measuring two fluorophores with different physical properties. For example, Elman and his associates15 recently investigated diffusional loss of fluorescein from the anterior chamber by measuring the simultaneous rate of disappearance of fluorescein (lost by outflow and diffusion) and blue dextran (lost only by outflow). Their analysis required that a sample of aqueous humor be removed at the end of the experiment to measure the blue dextran by absorption. The SOSF provides a means of performing a similar experiment without the need to remove aqueous humor. If fluorescein and a large molecular weight fluorophore such as rhodamine-labeled dextran were initially injected into the anterior chamber, their concentration could easily be measured over several hours and the simultaneous rate of disappearance be determined noninvasively. This scheme would allow repeated measurements for several hours after the initial injection.

The ability to measure fluorescence at selected excitation or emission wavelengths permits the exploitation of spectral properties of certain fluorophores. Fluorophores whose spectra vary as a function of their chemical milieu can be used as fluorescent probes of their microenvironment. For example, the ratio of fluorescence of fluorescein at 490 nm to fluorescence at shorter wavelengths changes with pH. Pyranine has the same property, exhibiting its major transition near neutral pH.16 By using differential fluorophotometry, one can take advantage of the pH dependence of the fluorophore and determine the pH of the tissue that contains the dye. This principle has been used to measure pH changes in cultured cells in vitro17 and recently in the living cornea.6 Many other fluorescent dyes show pH dependence and should be examined for their potential in measuring pH in vivo.18

Shifts of the entire spectrum of a fluorophore either toward longer or shorter wavelengths, as seen with fluorescein and rhodamine B in the cornea and lens, should reveal information about the composition of the tissues in which it is measured. Differences in spectral shift seen between humans and rabbits may reflect species differences in concentration or nature of albumins that bind fluorescein. By making the appropriate standardizations, one should be able to determine the concentration of a substance such as albumin by measuring the wavelength shift of the excitation or emission spectra of the fluorophore. Spectral changes of fluorescence in the cornea also illustrate the need for caution when determining concentration of a fluorophore in the eye from measured fluorescence. Fluorescence efficiency at a particular wavelength may not be the same in the cornea as in a simple buffered solution or aqueous humor. As a result, conversion of fluorescence to concentration may require different standardizations depending on the tissue that contains the fluorophore.

Some fluorescent molecules react to form fluorescent products that have different spectral properties from the parent compound. Resazurin, for example, is reduced in the presence of ascorbic acid to resorufin. Resazurin excites maximally at 600 nm and emits maximally at 630 nm; resorufin excites maximally at 570 nm and emits at 585 nm. These wavelength pairs can be measured separately to determine the rate of the reaction. In theory, by measuring the rate of conversion of this compound in the eye, one should be able to determine the concentration of ascorbic acid in the eye in vivo.8

These are a few examples of how scanning ocular
spectrofluorophotometry can be used to study ocular physiology in vivo. The ability to noninvasively measure spectral properties of fluorophores can reveal a good deal of information about the physical chemical state of the living eye that measuring only changes in fluorescence at a single wavelength cannot. The number of experiments is limited only by the availability of fluorescent probes with suitable properties and the imagination of the investigator.

**Key words:** fluorophotometer, spectrum, fluorescein, fluorescein glucuronide, rhodamine, resazurin

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**References**