High-Resolution, Noninvasive, Two-Photon Fluorescence Measurement of Molecular Concentrations in Corneal Tissue

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PURPOSE. To perform high-resolution, noninvasive, calibrated measurements of the concentrations and diffusion profiles of fluorescent molecules in the live cornea after topical application to the ocular surface.

METHODS. An 800-nm femtosecond laser was used to perform two-photon fluorescence (TPF) axial scanning measurements. Calibration solutions consisting of sodium fluorescein (Na-Fl; concentration range, 0.01%–2.5%) and riboflavin (concentration range, 0.0125%–0.1%) were tested in well slides, and TPF signals were assessed. Excised feline eyeballs preserved in corneal storage medium and with either intact or removed corneal epithelia were then treated with Na-Fl, riboflavin, or fluorescein dextran (Fl-d) of different molecular weight (MW) for 30 minutes. Calibrated TPF was then used immediately to measure the concentration of these molecules across the central corneal depth.

RESULTS. The axial resolution of our TPF system was 6 μm , and a linear relationship was observed between TPF signal and low concentrations of most fluorophores. Intact corneas treated with Na-Fl or riboflavin exhibited a detectable penetration depth of only approximately 20 μm , compared with approximately 400 to 600 μm when the epithelium was removed before fluorophore application. Peak concentrations for intact corneas were half those attained with epithelial removal. Debrided corneas treated with 2,000,000 MW Fl-d showed a half-maximum penetration depth of 156.7 μm compared with 384 μm for the 3,000 MW dextran. The peak concentration of the high MW dextran was one quarter that of the lower MW dextran.

CONCLUSIONS. TPF is an effective, high-resolution, noninvasive method of quantifying the diffusion and concentration of fluorescent molecules across the cornea. (*Invest Ophthalmol Vis Sci.* 2011;52:2556–2564) DOI:10.1167/iovs.10-6620

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Topical application of miotics, anti-inflammatory antibiotics, or molecular markers (e.g., fluorescein) to the ocular surface is a common means of assessing and treating a variety of pathologic ocular conditions. However, to date, we have no reliable method of quantifying how quickly topically applied molecules are able to diffuse across different corneal layers and what concentrations of these molecules are present at different corneal depths at given time points. Here we describe a new method for obtaining high-resolution, noninvasive measurements of molecular concentrations for fluorescent substances diffusing across the live cornea using two-photon fluorescence (TPF) imaging.

Conventionally, a slit lamp^{1,2} or a subjective grading system^{3,4} is used to qualitatively estimate the diffusion of substances across the cornea. Unfortunately, fluorophotometry and slit lamp approaches provide only limited axial resolution, typically hundreds of microns.⁵ With low axial resolution, any chemical's distribution in thin layers, such as the tear film (a few microns thick) or the corneal epithelium (tens of microns thick), cannot be resolved.⁶ Even penetration depth, concentration, or absorption dynamics in comparatively thicker structures such as the corneal stroma (several hundred microns thick) cannot be accurately determined. Confocal imaging can achieve micron-level axial resolution and has been applied extensively for studies of the cornea.^{7–9} Recently, it has been used to quantify the concentration and distribution of fluorescent molecules across this structure¹⁰; however, the linear attenuation of the exciting light across the corneal depth must be considered.¹¹ Two-photon excitation fluorescence offers distinct advantages over all these techniques in that there is essentially no linear absorption at the excitation wavelength, making it easier to obtain quantitative, 3D measurements.¹² With the use of near infrared wavelengths in the low absorption region of biological tissues, lower phototoxicity is obtained, allowing safe, long-term, light-matter interactions.^{13,14} Besides its inherent 3D submicron resolution,^{15,16} nonlinear microscopy demonstrates reduced bleaching and photodamage outside the focus point.¹⁷ The distribution of riboflavin in the corneal stroma of enucleated porcine eyes was recently measured with a similar TPF method,¹⁸ which had the advantage of being noninvasive and noncontact. However, the authors¹⁸ did not measure the precise concentration of riboflavin at different depths in the stroma or the resolution of the system.

Here, we describe a TPF system calibrated to quantify the distribution and concentration of fluorescent or fluorescently tagged chemicals and drugs in the live feline cornea with micron-level axial resolution. With this high-resolution method, we were able to measure, for the first time, both the penetration depth and the concentrations of molecules applied topically to the ocular surface, either with an intact or a removed epithelial layer. As a proof of concept, we tested two classes of fluorescent molecules—fluorescein and riboflavin—which are

commonly used in ophthalmologic practice. Fluorescein (λ_{abs} peaks ~ 480 nm, λ_{em} peaks ~ 525 nm) is a biocompatible chromophore used in clinical settings to assess the barrier function of the corneal epithelial layer,^{1,19} endothelial pathology,²⁰ and dry eye disease.^{21,22} Riboflavin, which has a reasonable two-photon absorption cross-section,²³ is raising a lot of clinical interest as a potentially useful drug (in conjunction with UVA exposure) for the treatment of keratoconus.²⁴ In this context, it is desirable to control the distribution and penetration of riboflavin in corneal tissue so as to optimize the efficacy and minimize toxicity of the UV treatment.²⁵ Finally, we used our TPF system to test the barrier function of the corneal epithelium and to measure the concentration of nonfluorescent molecules (in this case, dextrans), conjugated to fluorescent dyes, as they diffused across the cornea.

MATERIALS AND METHODS

Well Slide Phantom Preparation

Our first experiment was designed to measure the relationship between TPF signal and concentration of the fluorophores of interest. Na-Fl (MW 376.3; Sigma-Aldrich, St. Louis, MO) was diluted in corneal storage medium (Optisol-GS; Bausch & Lomb Inc., Rochester, NY) to achieve concentrations that ranged from 0.01% to 2.5%. A small drop of each Na-Fl solution was placed in the well (~ 0.4 -mm depth) of a dimpled microscope slide (OnlineScienceMall) and was covered with a glass coverslip (#1) before measurements were taken of TPF signals. Riboflavin (Seros Medical, Los Altos, CA) was dissolved in 0.02% benzalkonium chloride (BAK) and 100% D₂O to achieve a concentration of 0.1%, an osmolarity of 290 mOsm/mL $\pm$ 10 mOsm/mL, and a pH of 6.8 ± 0.2 . It was further diluted with distilled water to attain final concentrations that ranged from 0.0125% to 0.1%, placed in well slide phantoms, and covered with a glass coverslip for the purpose of measuring TPF signals.

Preparation of Doped, Live Enucleated Feline Globes

All animal procedures adhered to the guidelines of the University of Rochester Committee on Animal Research, the ARVO Statement for the Use of Animals in Ophthalmic and Vision Research, and the National Institutes of Health Guide to the Care and Use of Laboratory Animals.

Eighteen freshly enucleated feline eyeballs were purchased (Liberty Research Inc., Waverly, NY), placed in corneal storage medium (Optisol-GS; Bausch & Lomb Inc.), and shipped overnight on ice to our facility. This medium is a special one used to keep human corneas destined for transplantation alive for up to 14 days.^{26,27}

The epithelial layer on top of the cornea of each eye was either carefully removed or kept intact. The globe was then fixed onto a foam holder, with the cornea facing upward. A glass ring (Pfeiffer Glass Inc.) was placed on top of the cornea and filled with the fluorescent solution of interest (Fig. 1C). After doping for 30 minutes, the glass ring and the diffusing solution were removed, the globe was rinsed with fresh corneal storage medium, and TPF imaging was immediately performed.

The typically applied doping solutions were as follows: 0.375% Na-Fl dissolved in 10% dimethyl sulfoxide (DMSO; J.T. Baker, Phillipsburg, NJ) in lubricant eye drops (Celluvisc; Allergan, Inc., Irvine, CA); 0.1% riboflavin dissolved in 0.02% BAK and 100% D₂O; 1% 3000 MW fluorescein dextran (D3306; Invitrogen, Carlsbad, CA) and 1% 2000,000 MW fluorescein dextran (D7137; Invitrogen), both dissolved in 10% DMSO in Celluvisc. In all cases, the concentrations used were well below the point at which concentration quenching occurs (Fig. 2).

Optical Coherence Tomography Imaging of Enucleated Globes

A custom-built anterior segment optical coherence tomography (OCT) unit was used to image the enucleated globes, as previously described.²⁸ The thickness of the epithelial and stromal layers within the analysis area was estimated from the normalized backscatter intensity profiles extracted from the OCT images by measuring the distance between intensity peaks.²⁹

FIGURE 2. Calibration for fluorophores with their well slide samples. (A) Measurement of TPF signals versus sample depth for Na-FI in corneal storage medium. Na-FI concentrations ranged from 0.01% to 2.5%. (B) Measurement of TPF signals versus sample depth for riboflavin in BAK/D₂O in well slides. Riboflavin concentrations ranged from 0.0125% to 0.1%. TPF signals were relatively constant across well slide samples because the concentration of the fluorophore of interest was uniform for each sample. (C) Plot of TPF signal level versus concentration of Na-FI, generated from data in A. The TPF signal increased linearly as concentrations increased up to 0.375% and decreased afterward because of concentration quenching. (D) Plot of TPF signal level versus concentration of riboflavin, generated from data in (B). TPF signal increased almost linearly with concentrations from 0.0125% to 0.1% riboflavin in BAK/D₂O. Signals shown are the mean values with SD from three separate samples.

TPF Measurements

The light source used for all our measurements was a femtosecond-pulsed laser (MaiTai; Spectra-Physics; Newport, Santa Clara, CA) with an 80-MHz repetition rate, 100-femtosecond pulse width, and a tunable wavelength range between 780 and 920 nm. The power of the laser pulses was attenuated by a variable attenuator (Fig. 1A). The beam was then modulated by a chopper at 1.4 kHz and focused onto the sample or tissue using a water immersion objective (NA = 0.90, 60 $\times$, 2-mm working distance; LUMPlanFI; Olympus, Tokyo, Japan). The fluorescence from the sample was detected in epi-mode by a PIN photodiode (SGD-200; EG&G, URS Corp., San Francisco, CA) that was connected to a lock-in amplifier (SR830; Stanford Research Systems, Sunnyvale, CA) working at the modulation frequency. This modulation detection helped to achieve high detection sensitivity and background-free, low-noise measurements of the signals.^{30,31} The samples were mounted on a 3D scanning platform formed by three linear servo stages (VP-25XA; Newport). In the present study, only the z-axis stage was moved to perform the scan while achieving the TPF signal.

Fluorescence signals were measured as the samples were scanned continuously by a tight laser focus point. The TPF signals at each corresponding vertical sample position were recorded simultaneously with LabVIEW (National Instruments, Austin, TX). In this way, we measured the fluorescence signal as a function of scanning distance in the tissue.

For samples with known uniform concentrations such as well slide samples, the TPF signals were uniform when the laser focus was inside the samples (Figs. 2A, 2B). This TPF signal could then be plotted as a function of the corresponding concentrations, generating a calibration curve for each fluorophore. A bandpass filter at a wavelength of 600 nm, with a 40-nm bandwidth, was placed in front of the detector to reduce the effects of reabsorption for the fluorescence emitted from fluorescein.³² No bandpass filter was used for the riboflavin measurements.

For corneal samples, the TPF signal was measured as for the calibration procedure. Scanning was performed immediately after doping. The scanning process took <1 minute. We then compared the TPF signal level of the corneal samples with the calibration curves achieved from the well slide samples. This allowed us to calculate the concentration of the fluorophore of interest at each scanning point in the tissue. During the enucleated globe measurement, a coverslip (#1) was placed on top of the globe and fixed by binding to the holder. The whole sample was then transported to the stage, and the TPF scanning signal was acquired.

RESULTS

Depth Resolution of the TPF Measurement System

A thin layer (<1 μ m) of 0.375% Na-FI in corneal storage medium, sandwiched between a microscope slide and the coverslip, was scanned axially to characterize the system's axial resolution, as previously described.^{33,34} The axial resolution of system was measured as approximately 6 μ m (Fig. 1D), determined from the full-width-half-maximum (FWHM) of TPF signal.

TPF Signal Calibration of Na-FI/Riboflavin in Well Slides

Well slides filled with Na-FI were first measured with the TPF system (Fig. 1B). Uniform fluorescence signals were achieved for all the samples, with varying concentrations from 0.01% to 5% (Fig. 2A). When Na-FI concentrations increased from 0.01% to 0.375%, the TPF signal levels increased linearly. TPF signal started to drop for Na-FI concentrations above 0.375%^{35,36} (Fig. 2C).

Uniform fluorescence signals were also achieved in depth for well slides containing riboflavin solutions ranging in concentration from 0.0125% to 0.1% (Fig. 2B). TPF signals also increased nearly linearly over the riboflavin concentrations tested (Fig. 2D).

Using TPF to Measure Fluorophore Concentrations in Enucleated Feline Globes

Enucleated feline globes topically treated with different solutions for 30 minutes were placed in a custom-made foam holder (Fig. 1C) and were scanned to measure their TPF signal distribution. A large volume (~1 mL) of the topically applied solutions was held on the corneal surface by a glass O-ring for the full 30-minute application, allowing us to determine the concentration and diffusion profile of the molecules of interest. For convenience of comparison, we defined half-maximum penetration depth as the depth at which the TPF signal reached half its maximum value. Without particular mention, all quantitative results are averages values based on three separate measurements. TPF measurements on pure, undoped corneas were also performed to verify that no background signal was observed.

Globes topically treated with 0.375% Na-Fl in DMSO/Cel-luvisc were examined with either intact or removed epithelium, as were globes treated with 0.1% riboflavin in BAK/D₂O. All globes treated with intact epithelium were found to have TPF signals that dropped sharply after approximately 15 to 20 μ m depth, indicating a discontinuity of the penetration of Na-Fl (Fig. 3C) and riboflavin (Fig. 4C) and suggesting that the corneas had relatively intact epithelial barrier function. Peak concentrations of 0.047% Na-Fl and 0.028% riboflavin were mea-

sured within the epithelial layer of doped ocular globes. These represent 8-fold and 3.6-fold reductions, respectively, relative to the concentrations of Na-Fl and riboflavin applied topically to the surface of the eye.

For globes whose epithelial layer was removed, fluorophores could be detected through the entire stromal layer, a finding confirmed by measuring the thickness of this layer using OCT (Figs. 3E, 3F, 4E, 4F). The concentration of Na-Fl and riboflavin decreased gradually from the front stromal surface and achieved a half-maximum penetration depth of approximately 557 μ m and 415 μ m, respectively. From the OCT images (Figs. 3A, 3D, 4A, 4D), we also noted that the stromal layers of globes without epithelia were significantly thicker than the stromal layers of globes whose corneal epithelia were left intact. Descemet's membrane detachment was also observed in some globes without epithelia, suggesting a dysfunctional endothelium and endothelial pump function across the stroma. Average peak concentration of Na-Fl and riboflavin detected in denuded stroma were $0.131\% \pm 0.023\%$ and $0.041\% \pm 0.018\%$, respectively. These concentrations represented a 2.9-fold and a 2.4-fold reduction, respectively, relative to the concentrations of fluorophore applied to the front surface of the stroma. The large standard deviations on these measures are likely related to variability in endothelial function and corneal swelling between the different postmortem eyes.

In debrided globes treated with 1% Fl-d (D3306, D7137) in 10% DMSO/Cel-luvisc, the stromal layer was swollen to approximately 1-mm thick, and Descemet's membrane often detached from the posterior corneal surface, as seen in the OCT images (Figs. 5B, 5F). A half-maximum penetration depth of 384 μ m was measured for the 3000 MW Fl-d D3306, with a peak

FIGURE 3. Concentration distribution of Na-Fl in enucleated feline globes with and without an epithelial layer. Both globes were topically treated with 0.375% Na-Fl in 10% DMSO/Cel-luvisc. (A) OCT image of a topically treated feline globe with an intact epithelial layer. (B) Backscatter reflectivity profile achieved from (A), showing minimal swelling of the stromal layer. (C) Concentration profile achieved by depth scanning of TPF. Only 20 μ m (FWHM) was detected with measurable Na-Fl concentration. Compared with (B), this thickness was less than that of the epithelium, showing no significant penetration beyond the epithelium. (D) OCT image of topically treated cat eye globe with debrided epithelium. Note the significant swelling of the stromal layer and the detachment of Descemet's membrane. (E) Reflectivity profile achieved from (D). (F) Concentration profile achieved by depth scanning of TPF. Na-Fl could be detected throughout the entire, swollen, stromal layer (compare with E), with the concentration peaking at the front surface (~0.131% Na-Fl) and decreasing gradually toward the back surface of the cornea.

FIGURE 4. Concentration distribution of riboflavin in enucleated feline globes with and without an epithelial layer. Both globes were topically treated with 0.1% riboflavin in 0.02% BAK and 100% D₂O. The left globe was doped with an intact epithelium, and the right one was doped with the epithelium removed. (A) OCT image of a topically treated cat eye globe with an intact epithelial layer. (B) Backscatter reflectivity profile achieved from A, showing more significant swelling of the stromal layer than in Figure 3. This was likely due to longer tissue storage in corneal storage medium. (C) Concentration profile achieved by depth scanning of TPF. Measurable riboflavin concentration was detected down to only approximately 15 μ m below the ocular surface. Compared with (B), this thickness was less than that of the epithelium. No significant penetration is seen into the stroma. (D) OCT image of topically treated cat eye globe denuded of epithelium. Note the significant stromal swelling. (E) Reflectivity profile achieved from (D). (F) Concentration profile achieved by depth scanning of TPF. Riboflavin could be detected throughout the entire swollen stroma (compare with E), with the concentration peaking at the front surface (0.06% riboflavin) and decreasing gradually toward the back surface of the cornea.

dextran concentration of 0.407% (a 2.5-fold reduction compared with the applied concentration); half-maximum penetration depth for the 2000,000 MW Fl-d D7137 was 156.7 μ m, with a peak dextran concentration of 0.102%. This is roughly one-fourth that for D3306 and a nearly 10-fold reduction in concentration relative to the 1% solution applied to the ocular surface.

The half-maximum penetration depth and concentration results for all enucleated globes are summarized in Table 1.

DISCUSSION

The present study demonstrates that TPF can be used as a high-resolution tool for the measurement of chemical and drug distribution across the depth of corneal tissue. The axial resolution of our TPF system was found to be more than one order of magnitude higher than that previously reported using fluorophotometry.⁵ Furthermore, we show that TPF can be used to measure the concentration of molecules either as native fluorophores or as fluorophores conjugated to a molecule of interest.

Methodological Considerations

Several factors, such as absorption of the emission wavelength,³² lens aberrations,^{37,38} and scattering, can cause TPF signals to decrease as the laser scans through the medium being studied. We attained flat scanning signals for both Na-Fl and riboflavin in well slides, as long as fluorophore concentrations were $<0.375\%$ and $<0.1\%$, respectively. Therefore, at concentrations likely to be of clinical interest, our current

system is essentially free of these limitations, and fluorophore distributions in the cornea can be calculated accurately from measured TPF signals.

Concentration quenching can also cause TPF signals to drop at higher fluorophore concentrations.^{35,36} Although this phenomenon can be useful in molecular imaging,³⁹ it limited the measurable concentration range for our current system. More complex procedures (such as getting subsidiary information while measuring the TPF signal intensity and developing inverse algorithms to extract accurate concentration measures) would be needed in the future to compensate for concentration quenching.

A particularly attractive aspect of our TPF technique is that it is noninvasive and noncontact. As such, it is especially well suited for in vivo clinical and research applications. For in vivo applications in the eye, a major concern is safety, especially with regard to retinal exposure. The irradiance threshold on the retina for a similar laser source was reported^{3,40} to be 1900 ± 307 W/cm². Using an NA = 0.9 lens focused at the cornea in our current setup, with only 10 milliWatt (mW) laser power, the retinal irradiance is <10 mW/cm². Therefore, it is highly unlikely that the laser settings needed to perform TPF in the cornea, as described in the present study, would cause damage in the retina. In fact, nonlinear imaging has been performed in corneas of live animals with approximately 25 mW power and a similar laser source, and no tissue damage or photobleaching resulted.^{41,42} Finally, we should note that the sensitivity of our system could be further optimized by using photomultiplier detection.⁴¹ Such an approach would reduce the excitation power needed even further.

FIGURE 5. Concentration distribution of conjugated FL-d in enucleated, debrided feline globes. (A) Globe after 30 minutes of topical incubation with 1% dextran D7137 (MW 2000,000, containing 0.02% fluorescein). Note that there is no significant color change of the cornea, suggesting little penetration of the fluorophore into the stroma. (B) OCT image of cornea in (A). Note the swollen stromal layer and a detaching Descemet's membrane. (C) Backscatter reflectivity profile achieved from (B) and indicating that the central stromal thickness is approximately 850 μm . (D) Concentration profile of FL-d D7137 achieved by depth scanning of TPF. Although the dextran was likely present across most of the stroma and exhibited the typical pattern seen in Figures 3 and 4, of higher concentrations at the front surface of the stroma, decreasing gradually more deeply into the cornea, the peak concentrations reached for this large molecule were very low ($<0.1\%$). (E) Debrided globe after 30 minutes of topical incubation with 1% dextran D3306 (MW 3000, containing 0.125% fluorescein). Note the corneal color change toward yellow, indicating significant penetration of this smaller conjugated fluorophore into the stroma. (F) OCT image of cornea in (E), showing a significantly swollen stromal layer and a detaching Descemet's membrane. (G) Reflectivity profile achieved from (F), indicating an approximate central stromal thickness of 1000 μm . (H) Concentration profile of FL-d D3306 achieved by depth scanning of TPF. This smaller dextran diffused across the entire corneal depth, with a peak concentration of approximately 0.36% at the stromal front surface, which decreased gradually to almost zero at the endothelial surface.

TPF, a High-Resolution Tool for the Study of Epithelial Barrier Function

The present results show that the axial resolution of TPF is sufficient to resolve the diffusion of molecules across the epithelial layer of the cornea. When Na-Fl and riboflavin were applied topically to feline eyeballs with intact epithelia and allowed to sit at room temperature for 30 minutes, the TPF signal was detected approximately only 20 μm below the ocular surface. This is much smaller than the thickness of the epithelial layer on those globes, measured with OCT (Table 1;

Figs. 3, 4). Thus, even though the corneal surfaces were bathed in chromophore for 30 minutes, no significant penetration of Na-Fl or riboflavin was observed beyond the corneal epithelium and into the stroma.

Primarily because of tight junctions between epithelial cells, the corneal epithelium's barrier function prevents macromolecules (molecular weight >182 Da) from entering the stroma.⁴³ This barrier function has usually been studied with fluorophotometry, which, as mentioned earlier, has only sub-millimeter resolution.⁴⁴ We now provide evidence that a high-

TABLE 1. Penetration Depth Comparison of Molecules into Corneal Tissue of Enucleated Feline Globes

	Chemicals/Drugs					
	Na-Fl		Riboflavin		Fl-Dextran D3306	Fl-Dextran D7137
Molecular weight	376.3		376.4		3,000	2,000,000
Fluorophore concentration	0.375% in 10% DMSO/Celluvisc		0.1% in 0.02% BAC and 100% D ₂ O		1% in 10% DMSO/Celluvisc	
Doping time	30 min		30 min		30 min	
Epithelium	Intact	Removed	Intact	Removed	Removed	Removed
Epithelium/stroma thickness, μm (SD)	78.7 (6.4)/633.7 (52.9)	NA/868.7 (73.1)	55 (8.7)/982 (54)	NA/1006 (118.2)	NA/962.5 (30.8)	NA/1045.3 (47.9)
Half-maximum penetration depth,* μm (SD)	19.7 (4.9)	557 (64.1)	19 (5.3)	415.3 (93)	384 (49.9)	156.7 (20.9)
Peak concentration, % (SD)	0.047 (0.015)	0.131 (0.023)	0.028 (0.002)	0.041 (0.018)	0.407 (0.028)	0.102 (0.017)

* Depth at which concentration reached half its maximum value at the front surface.

resolution tool such as TPF may provide more accurate measurement of molecular penetration across the cornea's epithelial barrier. As such, this may be an ideal technology with which to study, among other things, the recovery of epithelial barrier function after epithelial (and corneal) wounding.⁴⁵

TPF, a New Tool for the Quantitative Assessment of Ocular Drug Delivery

A potential area of applicability for noninvasive TPF measurements is in ocular drug delivery. Here, we measured the diffusion of Na-Fl, a chromophore commonly used in ophthalmic practice, during a 30-minute application in corneas both with and without an epithelial layer. Na-Fl was diluted into a solution consisting of 10% DMSO in Celluvisc to promote easy and rapid penetration of Na-Fl across the entire corneal thickness, so as to test the sensitivity of our TPF system at imaging this fluorophore at depth. Full penetration of Na-Fl through the stromal layer was shown by contrasting the TPF results with those of OCT imaging of the corneal thickness. Peak concentration was found at the front surface of the cornea, as shown previously in a slit lamp study,² but with more than one order of magnitude higher resolution. Given that the dosage and formulation applied in the two studies were different, however, quantitative comparison of the concentration distribution results is not possible.

We then tested the feasibility of our approach on riboflavin, a drug in limited clinical use for the treatment of keratoconus.⁴⁶ We showed riboflavin to have a significant, native, two-photon absorption cross-section and found a linear relationship between its TPF signal and concentrations up to 0.1%. Therefore, we were able to easily map the axial distribution and concentration of riboflavin after a 30-minute topical application to the corneal surface. Peak concentration was achieved at the front surface of the stroma, averaging $0.041\% \pm 0.018\%$ (Table 1). As for the Na-Fl diffusion experiments, the relatively large SD might have come from variability in endothelial function among the different eyes tested. Our peak value was also lower than that reported in the porcine cornea,⁴⁷ possibly because of our having rinsed the corneal surface with corneal storage medium after riboflavin application. It could also have been due to different tissue preparation methods and possible species differences in corneal diffusion.

We note here that different formulations of both Na-Fl and riboflavin may cause differences in penetration and concentration as they cross the cornea. As mentioned earlier, the formulations used in the present study were not the same as most current solutions designed for clinical use. For

instance, our riboflavin solution did not contain dextran, as used in other studies.^{10,18,47} However it was also intended as a potential recipe for cross-linking. Dextran containing riboflavin may de-swell an already thinned cornea. Non-dextran-containing formulations have been used to allow thin corneas to swell, theoretically decreasing the risk of damaging the corneal endothelium. The advantages of each must be further evaluated. Additional experiments must be performed to assess the impact of chemical composition (including dextran), osmolarity, pH, application time, and delivery method on diffusion of different drugs into the cornea. Our TPF method is ideally suited to perform these experiments because it has the capacity to measure diffusion rate and concentration in situ. We also speculate that other ocular drugs with reasonable two-photon absorption cross-sections could be measured directly using TPF. Not until we understand how chemistry, osmolarity, pH, application time, and delivery affect diffusion rate and concentration in the living, in situ cornea can we make recommendations about optimal formulations for different clinical applications.

When the drug of interest lacks a measurable two-photon absorption cross-section, it may still be possible to use TPF to measure its concentration and distribution in ocular tissue by conjugating the drug to a two-photon fluorophore. For example, the uptake of folic acid has been examined in kidney proximal tubules by tracking fluorescein and Texas Red conjugates of folate with two-photon microscopy.⁴⁸ Here, as a proof-of-concept experiment, we measured the diffusion profile and concentration of dextrans conjugated to fluorescein across the corneal stroma after topical application to the debrided ocular surface. The 2000,000 MW Fl-d D7137 showed significantly less penetration and a lower peak tissue concentration than the 3000 MW Fl-d 3306. This suggests that large molecular weights significantly reduce diffusion across stromal layers. With the development of new conjugate fluorophores,⁴⁹ the diffusion of many ocular drugs, such as miotics, anti-inflammatory agents, and antibiotics, across the cornea could be quantified for the first time. We note that when choosing a fluorophore for conjugation to ocular drugs of interest, one should be careful with its toxicity, molecular weight, and potential for interfering with each drug's effectiveness.

As we stated in Results, stromal swelling was observed after epithelial debridement. More severe corneal swelling was observed in riboflavin-treated corneas, something we feel is related to the fact that those globes were kept for approximately 40 hours longer in corneal storage medium than those treated

with Na-Fl. Such prolonged storage likely impaired the endothelial pump's function (as evidenced by detachment of the Descemet's membrane and the endothelial layer shown in Fig. 4), resulting in greater corneal swelling.⁵⁰ As corneas swelled, their thickness and the doping concentration distribution were also changing over time. However, OCT images and TPF measurements, which were performed within minutes of each other, showed extremely close values for corneal thickness. Therefore, we do not believe this was problematic for our results.

CONCLUSIONS

Here we present a new TPF method for measuring the concentration of fluorescent molecules as they diffuse across the cornea. This new method has a depth resolution of 6 μm , which is significantly higher than that of traditional fluorophotometry. Calibration of Na-Fl solutions was achieved and showed TPF signals linearly related to concentrations when the concentrations were low.

When Na-Fl or riboflavin was applied topically to the corneal surface of enucleated globes and held in place for 30 minutes, no penetration of either fluorophore was observed beyond the epithelial layer. However, if the epithelial layer was removed, measurable concentrations of both fluorophores were mapped across the entire stromal layer. Increased molecular weight decreased the rate of diffusion across the stroma and the peak concentrations achieved. Overall, the present results suggest that TPF can be used as an effective, noninvasive, high-resolution method for measuring the distribution and concentration of both native fluorophores and fluorescently tagged molecules across the living cornea. Future studies will systematically examine the effects of chemical composition (i.e., formulation), osmolarity, pH, topical application time, and delivery method on the diffusion of different drugs into the living, in situ cornea. Results from these studies will allow us to predict optimal formulations of different drugs for clinical application in the treatment of ocular disorders.

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