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Fluorescence lifetime imaging microscopy reveals quenching of fluorescein within corneal epithelium

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Abstract

Topical application of fluorescein results in background fluorescence of normal corneal epithelial cells. The fluorescence appears relatively weak and is often ignored clinically. The concentrations of fluorescein applied clinically exceed the threshold for self quenching. The possibility that exuberant topical concentrations of fluorescein result in quenching of fluorescence in tears and normal corneal epithelium is explored. Fluorescence lifetime measurements are sensitive to quenching and are less vulnerable to inner filter effect than steady state measurements. The types of fluorescence lifetime quenching often report informative molecular interactions. Therefore, fluorescence lifetime confocal imaging was performed in solutions, tears and corneal epithelium removed by membrane cytology following applied fluorescein. Amplitude averaged fluorescence lifetimes (τ_{amp}) were measured with time resolved single photon counting using a pulsed diode laser for excitation of fluorescein. Lifetime decays were fit to multi-exponential models with least squares analysis. Stern-Volmer plots for both intensity (I) and (τ_{amp}) were determined. Stern-Volmer plots demonstrated both dynamic and static quenching components ($R^2 = 0.98$ exponential fit, I_0/I). Plots of τ_{amp} versus concentration of fluorescein revealed a linear relationship. Immediately after fluorescein application, quenching was evident in tears ($\tau_{amp} < 1$ ns) versus tears sampled after 5 min ($\tau_{amp} = 3.7$ ns). Corneal epithelium showed quenching ($\tau_{amp} \geq 2$ ns) from 1 to 16 min post fluorescein instillation. Clinical concentrations of fluorescein show self-quenching but rapidly dilute as tears turnover. Intracellular quenching occurs in normal corneal epithelium. Lifetime decay curves suggest complex mechanisms are involved. Quenching is a plausible explanation for the low fluorescence background observed clinically.

Keywords

Corneal epithelium; Fluorescence lifetime; Tears; Fluorophore quenching; Fluorescein staining of epithelium

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1. Introduction

Nearly all normal subjects demonstrate some corneal epithelial staining of fluorescein even under relatively weak excitation from the ordinary slit lamp and blue filter (Dundas et al., 2001; Schwallie et al., 1997; Yokoi et al., 1996). Although faint background fluores-cence is generally ignored at the slit lamp, virtually every corneal epithelial cell fluoresces shortly after application with fluorescein when viewed under a fluorescent microscope (Feenstra and Tseng, 1992; Gorbet et al., 2014; Mokhtarzadeh et al., 2011; Thinda et al., 2010; Wilson et al., 1995). Penetration of fluorescein at various levels of the cornea has been described (McNamara et al., 1997; Webber and Jones, 1986). Despite this abundant evidence the notion seems to persist that fluorescein does not enter normal corneal epithelium with intact desmosomes (Bron et al., 2015; Romanchuk, 1982). The mechanism of entry of fluorescein into corneal cells is not established and the topic is controversial (Bron et al., 2015; Mokhtarzadeh et al., 2011). Obfuscating the mechanism is the wide range of fluorescein concentrations used clinically (up to 1%). These concentrations exceed even conservatively high estimates for the expected threshold for self-quenching (Doughty, 2010; Luchowski et al., 2010; Nichols et al., 2012; Romanchuk, 1982; Van Duuren, 1961; Wessing, 1969). Many past studies are hampered by inner filter effects, Some have recommended a minimal concentration, 0.125% to avoid fluorescein self-quenching (Bron et al., 2015; Norn, 1970). Still that concentration surpasses the threshold for self-quenching that many have measured (Norn, 1970; Umberger and La Mer, 1945).

Characterizing the intracellular fluorescence of normal cornea cells is critical to discerning the mechanism of fluorescein staining for diagnostic use. The possibility of intracellular quenching of fluorescein in normal corneal epithelium has not been experimentally investigated. Uncalibrated single intensity measurements in cells do not provide a measure of molecular quenching. But a single time-resolved fluorescence lifetime measurement accurately identifies quenching of fluorophores and is less susceptible to inner filter effects. The purpose here is to determine whether fluorescein in clinically applied solutions self-quenches and if quenching occurs within the corneal epithelial cells by fluorescence lifetime measurements.

2. Materials and methods

2.1. Fluorescein

Fluorescein, molecular weight 376.2 g/mole, was obtained for stock solutions from sterile fluorescein impregnated strips (Bio Glo strips HUB Pharmaceuticals, LLC, Rancho Cucomonga, CA). Instilled drops were diluted in sterile sodium phosphate buffer pH 7.5 and checked and adjusted gravimetrically to minimize systematic pipetting errors. For all experiments the concentration of sodium fluorescein was determined by absorption spectrophotometry (Shimadzu UV-2401PC, Kyoto, Japan) after dilution to the range of about 15 μM and adjustment to pH 9 using the molar extinction coefficient as previously described (Sjöback et al., 1995; Yeh et al., 2013).

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2.2. Time-resolved fluorescence microscopy measurements

Time correlated single photon counting fluorescence lifetime confocal imaging was performed with the MicroTime 200 system (Picoquant Inc. GmbH, Berlin, Germany). A single mode optical fiber captured pulse diode laser excitation λ = 485 nm and was triggered by a dedicated picosecond Sepia II laser driver. The signal was synchronized with the Pico Harp 300 event timer and photon counting module. The excitation beam was focused through a high numerical aperture (1.2 water immersion objective (Olympus) to the samples that were placed between or on 170 μm thickness coverslips. The maximum path length or distance between cover-slips was estimated with the calibrated microscope z stage. For droplets the maximum height (h) or pathlength was calculated from modification of the standard formula for the volume of a spherical cap:

$$v=\frac{1}{3}h^2\pi * (3r-h)$$

where the average radius, r, was consistently 2 mm with a maximum volume (v) of 7 μL. To further minimize the path length, the objective was focused on the closest coverslip at the surface near the sample using the interference fringes of the reflected light. Scanning was performed with a digitally controlled piezo-scanner (model P-733.2CL, Physik Instrumente (PI) GmbH Co., Karlsruhe, Germany). Fluorescence emission was collected through the objective, passed through a dichroic mirror 500 DCXR (Chroma Technology Corp. Bellows Falls, Vermont) focused into a pinhole aperture, filtered through a band pass filter 525/45 (Semrock) and detected by a single photon avalanche photodetector (SPAD) (Micro Photon Devices, Bolzano, Italy). Lifetime decay curves were fit to a multi-exponential model with SymPhoTime v. 5.0 software (Pico-Quant, Inc. GmbH, Berlin). The amplitude average lifetime τ_{amp} was calculated:

$$\tau_{amp}=\sum_i \alpha_i \cdot \tau_i$$

where α refers to amplitude fractions and

$$\sum \alpha_i=1$$

Steady state intensity was derived from the total integrated photon count and provided by the manufacturer software (Sym-PhoTime v. 5.0).

For determining the bimolecular quenching constant, k_q, was computed using the Stern-Volmer constant, $K_{SV,}$ as previously described (Lakowicz, 2007):

$$k_q=K_{sv}\tau_0$$

K_{SV} was derived from the slope of Stern-Volmer plot of τ_o/τ versus the quenching concentration.

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Because serially diluted concentrations measured by gravimetric analysis are not identical, the lifetime results were analyzed as means of binned concentrations in order to compare threshold of intensity versus lifetimes. The lifetime data were also fit from a scatter plot of τ_{amp} vs concentration to determine the function from a more precise data set. Correlation coefficients, R^2, were calculated using least squares regression analysis.

2.3. Human subjects for study of corneal epithelium

This study was performed in accordance with the Institutional Review Board of the University of California, Los Angeles. Written informed consent was obtained from all subjects involved, after explanation of the nature and possible consequences of the study. The research adhered to the tenets of the Declaration of Helsinki. Two normal subjects were recruited for ocular impression cytology after the diagnosis of dry eye was excluded as previously described (Mokhtarzadeh et al., 2011).

2.4. Lifetime measurement of fluorescein in tears

Fluorescent lifetimes of fluorescein control solutions were measured to choose a non-quenching concentration, ~26 μM, as a negative control I_0 and τ_0 for self quenching. 22 mM solutions were compared in tears at baseline and 5 min after instillation. The steady state intensity was measured as the total integrated photon count from the Picquant Microtime 200 software.

2.5. Cytology with glass membranes

To capture mainly single cells for fluorescence lifetime imaging, and avoid autofluorescence inherent in many cytology membranes, thin strips of cover glass were fashioned into a tiny membrane. Cover glass was fire polished and shaped with a handle to provide a smooth small area of contact with the cornea (1–2 mm^2) that was easily controlled. The glass membrane was pressed gently against a small area of the cornea as previously described (Mokhtarzadeh et al., 2011; Yeh et al., 2013). To avoid the possibility of incidental diffusion of fluorescein between lateral cell surfaces, the procedure was performed only once for each single eye. For precise delivery, 7 μl of fluorescein, 22 mM, was pipetted in the inferior cul de sac for clinically relevant cytology experiments, which would result in a maximum of 11 mM fluorescein concentration in tears, within the range produced by impregnated strips (Abdul-Fattah et al., 2002). Unstained corneal cells were examined to ensure an absence of background fluorescence and scattered light.

3. Results and discussion

The key findings in these results are: 1. fluorescein at clinically employed concentrations self-quench, 2. the self quenching mechanism of fluorescein in solution is complicated and involves static and dynamic components, including-homo resonance energy transfer, and 3. fluorescein applied to the ocular surface at clinically used concentrations, remain quenched within epithelial cells beyond the time of routine clinical examination, 16 min.

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3.1. Instrument response function

Applied concentrations of sodium fluorescein for diagnostic use on the ocular surface vary widely in the literature from 0.125% (3.3 mM) (Norn, 1970) – 2% (53 mM) (Korb and Korb, 1970). The threshold at which fluorescein concentration dependent reduction of fluorescence has been reported is also highly variable ranging from 98 to 53,000 μM (Table 1). The sources of variation in the measurements are legion and include the mode and duration of excitation, optical pathway (beam splitters, dichroics, filters and detectors, beam geometry), as well as the experimental conditions such as sample thickness, pH, inaccuracies in serial dilution etc (Lakowicz, 2007) (Luchowski et al., 2010). In some cases the measurements in Table 1 may reflect predominantly a threshold for inner filter effects, attenuation of both absorbed and emitted light. In two reports (Maurice, 1967), and (Webber and Jones, 1986) a threshold of fluorescence reduction was measured directly on the tear film, presumably 5 μm in thickness. The threshold concentrations of fluorescein were calculated and far exceed all other reported quenching threshold concentrations for fluorescein. It is not clear why these experiments were insensitive to quenching. Perhaps the high concentrations reflect the threshold for reduction of fluorescence due to inner filter effects rather than true quenching. In thin samples the inner filter effect becomes apparent only at very high concentrations. This concept was utilized in our study to eliminate inner filter effects by decreasing the path length for intensity measurements. Variability in fluorescence measurements may occur even in a single experiment with a wide range of fluo-rescence intensities that requires adjustment of neutral density filters to achieve an appropriate signal for sensitive detectors. Day to day variations in laser power and optical alignment add to the inconsistencies. A wide range for thresholds is expected as measures of fluorescence are relative and vary according to the type of fluorescent measurement (i.e steady state, anisotropy, fluorescence lifetime). As in the present study, fluorescence is often measured at concentration intervals. Quenching may begin at a concentration between those chosen for the interval. Greater precision was not required in this case as the quenching threshold was needed for calculations of I_0 and τ_0 in Stern-Volmer plots, and to characterize the other overall instrument response function as well as choosing control concentrations for lifetime imaging. Dilute fluorescein was the main calibrant for fluorescence lifetimes, which at about 4 ns matches published values in the literature and corresponds to τ_o (Luchowski et al., 2010).

3.2. Self-quenching of fluorescein

Characterization of fluorescein self-quenching in solution by steady state and lifetime fluorescence is critically important to interpreting biologic τ_0 samples. Despite the variation in instrument response function, concentration dependent reduction of fluorescence of fluorescein can be easily visualized from a charge coupled device camera (Fig. 1). Because the samples are interposed between the light source and the CCD, the entire path length, ~1 mm, of the droplet is utilized. Inspection of Fig.1 shows an initial rise in steady state fluorescence intensity with increasing fluores-cein concentration. Brightness appears to reach the maximum for the spots in the range matching the peak shown in Fig. 2 (about 2048 μM). Thereafter (Fig. 1), the spots decrease in brightness and show a dark green color. These changes are consistent with self-quenching. A significant inner filter effect is unlikely (Fig. 1). Fluo-rophores with a small Stokes shift (overlap of absorbance and emission spectra) generally show a red shift due to reabsorption of the emitted shorter wavelengths. With an

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inner filter effect a red shift or red color is expected rather than the observed dark green. These findings are in line with the steady state fluorescence intensities threshold measured from the total photon count in the Picoquant system in repeated experiments (Fig. 2) and in the range of published results (Table 1) (Luchowski et al., 2010). The threshold concentration was considered the peak when fluorescence intensity begins to decrease with increasing concentration in the steady state measures. For lifetimes, the unquenched samples are identical so the threshold for quenching was chosen for the concentration where the mean lifetime from multiple samples differed significantly from the τ_0. The values were determined for both steady state fluorescence and fluorescence lifetimes of the same samples. The fluorescence lifetimes appear more sensitive to self quenching; measured lifetimes diminish even as intensity continues to rise (Fig. 2). Despite some variability in the threshold for self quenching, a conservatively high 500 μM as determined from lifetime measurements is very close to early published results in solution (Pringsheim,1949), and roughly in line with that published for fluorescein in a solution of polyvinyl alcohol that was spin coated to a solid film (Table 1 and Fig. 2) (Luchowski et al., 2010). The lifetime quenching threshold of 500 μM is a conservative overestimate because the criterion for threshold was a significant difference calculated from the means of several measurements. In several individual experiments, reduced fluorescence lifetimes were observed at ~100 μM fluorescein but the changes were small and statistically insignificant when the means were compiled. This is evident in Fig. 2 as the initial fall in lifetimes is gradual and the variation in lifetimes is reasonable when fluorescein is dilute. Fluorescence lifetimes are maximum and constant for dilute solutions so a small dilution error will have little effect. However for more concentrated solutions, errors in preparation of accurate serial dilutions affect lifetime measurement. Instrument induced pipetting errors are often systematic and are multiplicative in numerous serial dilutions. Many serial dilutions are needed to dilute fluorescein to determine a reasonable absorbance value. Errors in measurement of concentration may be underestimated and account for some reported fluorescein quenching thresholds that differ by orders of magnitude (Table 1). In the experiments reported here, the mean threshold concentration for fluorescence quenching was about 4 fold greater for steady state intensity than lifetime measurements (Table 1, Fig. 2). There are several possible explanations. Inner filter effects are possible but were mitigated by reduction of the maximum possible path length to 170 μm for samples between coverslips and to 1 mm for the spherical cap height (path length for droplets). The effective path lengths were further reduced by confocal microscopy using focus near the objective side of the sample surface and the inherent frontal geometry. These changes have been shown to minimize the inner filter effects (Lakowicz, 2007) (Rohatgi and Singhal, 1962). For the steady state measurements the alterations to reduce path length appear effective as fluorescence intensity rises when the lifetimes fall and no significant differences in intensity were detected between droplet and solutions between coverslips. Fortunately fluo-rescence lifetime measurements are generally independent of inner filter effects (Lakowicz, 2007). However, in theory it is possible in thick samples that reabsorption of emission followed by re-emission could prolong the lifetime, but this phenomenon was not observed in these experiments. Notwithstanding the gaps between concentrations and the relatively large error involved at each point, the disparity can be further rationalized. As fluorescein molecules are added just below the quenching threshold the majority of the added population is unquenched. The additional unquenched

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fluorophores raise the intensity but the unquenched population shows the maximum lifetime value. The first quenched species will show an immediate reduction in the ensemble of average lifetime whereas intensity may continue to rise until quenched species predominate.

3.3. Fluorescein concentrations in clinical use are self-quenching

The initial concentrations of fluorescein instilled on the cornea are at least one order of magnitude above the threshold for true self-quenching reported here and in the literature (Table 1) with the exceptions discussed in Section 3.1. A convenient procedure extolled in a thorough review recommended touching a fluorescein impregnated strip applicator to the conjunctiva with the residual left after a drop of saline was shaken off (Bron et al., 2015). The minimum amount delivered as reported from the most conservative approach of this method is 0.016 mg (Abdul-Fattah et al., 2002). The final concentration expected in the tear film which has a volume of 7 μl tear film is still 6 mM. Depending on the area of the strip wetted, the range of concentrations resulting in tears was 6–27.7 mM, well above self-quenching thresholds (Abdul-Fattah et al., 2002). None of the methods of fluorescein application, in particular the strip applicator, is likely to deliver or result in precise concentrations of fluorescein in tears since in practice a crude (filter paper) dispenser is used and variable amounts of tear fluid exist in the cul de sac. Even with the variations and discrepancies in technique the longevity and apparent usefulness of self-quenching concentrations of fluorescein suggest exceptional forgiveness of error.

3.4. Mechanisms of self-quenching

The mechanisms of self quenching of fluorescein have been variably reported to involve polymer formation, resonance energy transfer and self-absorption. The Stern-Volmer plots of both lifetime and intensity measurements (Fig. 3) demonstrate the complexity. In the concentration range of interest the intensity plot (Io/I) fits an upward sloping exponential curve and that is consistent with both static and dynamic quenching mechanisms (Lakowicz, 2007). Fluorescein molecules interact to form dimers and polymers in solution (Arbeloa, 1981a, 1981b; Lavorel, 1957; Pant and Pant, 1968). These complexes have been reported as non-fluorescent (Bowen, 1970; Van Duuren, 1961) and explain the apparent static quenching evident in the Stern-Volmer plot. The influence of static quenching appears dominant over dynamic quenching at high concentrations of fluorescein as I_0/I greatly exceeds τ_0/τ.

Because the data in Fig. 2 were binned to generate approximate means for comparison of thresholds, the scatter plot of fluorescence lifetime may render more precision for determining the mathematical relationship of τ vs concentration (Fig. 4). A linear relationship is approximated ($R^2 = 0.92$). This finding has been reported by others in self-quenching experiments of rhodamine 6G, which is structurally similar to fluorescein (Penzkofer and Lu, 1986; Selanger et al., 1977). The Stern-Volmer plot is essentially the inverse of τ versus concentration. If the latter is linear then τ_0/τ versus concentration describes a hyperbola. Interestingly, the Stern-Volmer plot also approaches a linear fit in this range, $R^2 = 0.94$. This is possible if the line approaches the tangent to the hyperbola. The slope of this line can be used to approximate the Stern-Volmer constant or dynamic quenching constant, then the subsequently calculated bimolecular quenching constant, 3.2×10^{10} M^{-1} sec^{-1}, is 3 fold greater than the maximum diffusion rate in water, 1×10^{10} M^{-1}

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sec^{-1}. This impossibility precludes classical collisional quenching as the sole mechanism (Lakowicz, 2007). Additional mechanisms must be invoked to explain dynamic quenching. Particularly insightful are experiments done with fluorescein spun in polyvinyl alcohol thin films (Luchowski et al., 2010). A "sublinear" plot is described for increasing concentration versus lifetime measurements. A maximum limit is approached for τ_0/τ. The implication is that a residual fluorescent species exists at high concentrations. In solid phase, collisions of molecules are precluded so that dynamic quenching is much more likely due to a near field effect or homo-resonance energy transfer. Such an explanation is consonant with the additional component of dynamic quenching in solution observed in the current study. Luchowski et al. also demonstrated that in spin coat film preparations increasing concentrations of fluorescein were associated with decreasing anisotropy. Depolarization from rotation of molecules is excluded in the solid state, implicating resonance energy transfer from adjacent dipoles. One possibility is that fluorescein forms dimers, trimers and polymers, some of which do not remain completely in the ground state after excitation. The complex mechanism of fluorescein self-quenching may include a combination of dynamically quenched complexes from homo-RET as well as static quenching from non-fluorescent ground state complexes. An additional component of collisional quenching cannot be excluded. Regardless of the mechanism it is clear that self-quenching occurs in fluorescein solutions at the very high concentrations instilled on the eye to identify ocular surface disease.

3.5. Fluorescence quenching of fluorescein in tears and corneal epithelium

Fluorescein quenching was further explored in tears. Immediately after instillation of 22 mM fluorescein, the fluorescence lifetime of tears was found to be about 1.2 ns (mean of n = 3). After 5 min the samples of tears show a lifetime of 3.7 ns with an associated 7 fold increase in intensity. The apparent release of self-quenching of fluorescence can probably be attributed to rapid tear turnover. Tear turnover is generally measured with fluorescein and has been as reported to decay in the first order with the range of 7–60% per minute depending on the methods used (King-Smith et al., 2013; Mishima et al., 1966; Nichols et al., 2012; Occhipinti et al., 1988; Webber and Jones, 1986; Webber et al., 1987). In the data presented here, an approximate minimum 4 fold reduction in concentration in 5 min is expected from the increase in τ_{amp} (Fig. 2) and falls within the mid-range predicted from the published tear turnover rates (Occhipinti et al., 1988). Interestingly, the tear turnover rate has been published to fall with less concentrated fluorescein attributable to a contribution from persistent corneal fluorescence (King-Smith et al., 2013). In fluorophotometry experiments (de Kruijf et al., 1987; McNamara et al., 1997), fluorescence persists for 15–20 min despite tear washout (Gorbet et al., 2014; Joshi et al., 1996). The degree to which the fluorescence emanates from the epithelium as opposed to the stroma is not established.

All corneal cells removed for cytology after fluorescein instillation showed fluorescence and quenching. After 5 min and 16 min the means ± standard deviations of measured average lifetimes were (0.84 ± 0.31 ns) n = 4 and (1.4 ± 0.6 ns) n = 12, respectively. A representative experiment to study intracellular quenching of fluorescein in corneal epithelial cells is shown in Figs. 5–7. Individual cells show fluorescence average lifetimes ranging from 0.4 to ≤ 2 ns (Fig. 6). The relative intensity of the cells is far greater than the background (Fig. 5).

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The lack of fluorescence outside the cells indicates the cell membranes are intact. Further, the dark background indicates a paucity of noise. Corneal cells in the absence of fluorescein showed no signal (negative control for scatter and autofluorescence). Fluorescence remains quenched in cells collected 5–16 min after instillation of fluorescein (Fig. 6). Quenching persists despite the fact that the concentration of fluo-rescein in tears has decreased beneath the quenching threshold at this time point. Simple rapid diffusion as the mechanism of fluo-rescein exiting the cells is unlikely. Rather the mechanism of intracellular quenching appears much more complex. The lifetime decay could not be fit to a mono-exponential model (Fig. 7). Intraepithelial quenching of fluorescein may involve homo-resonance energy transfer and be complicated by binding to membrane transporters or intracellular proteins. The uptake of fluorescein within cells may depend on the concentration of fluo-rescein instilled. Although not studied in detail here, the signal was insufficient for reliable lifetime imaging below 20 nM. Fluorescein has been reported to enter epithelial cells including those of the cornea via monocarboxylate transporters (Chidlow et al., 2005; Konishi et al., 2003; Kuwayama et al., 2002; Vellonen et al., 2010). Fluorescein has been shown to bind proteins, specifically albumin and immunoglobulins (Herron et al., 1989; Li and Rockey, 1982; Penniston, 1982; Rockey et al., 1983). Albumin has been identified by mass spectrometry in normal corneal epithelium (Joseph et al., 2011). The dissociation constant for fluorescein bound to human albumin has been estimated to range from 0.320 to 410 μM with about 4 molecules of fluorophore bound to each molecule of albumin (Nagataki and Matsunaga, 1985; Penniston, 1982). Multiple fluorophores bound in proximity on the same protein often show quenching (Lakowicz, 2007). So it is not surprising fluorescein bound to albumin demonstrates quenching, both static and dynamic (Barbero et al., 2009; Kinoshita et al.,1987). Fluorescein entry into human lymphocytes has been proffered to exist in both bound and free states (Meisingset and Steen, 1981). The bound fraction predominates, involves many intracellular proteins and shows increased anisotropy (Kinoshita et al., 1987; Meisingset and Steen, 1981). These data are consonant with the findings presented here for corneal epithelial cells. The overall impression is that quenching from intracellular protein binding is likely but self-quenching has not been excluded as the concentrations used clinically for the eye are orders of magnitude higher than those used in the studies of lymphocytes.

3.6. Implications of fluorescein quenching in normal epithelium

The quenching of fluorescence in normal corneal epithelial cells has implications for the punctate staining pattern of fluorescent epithelial cells of the cornea, a key diagnostic criterion for dry eye disease (Nichols et al., 2011), (Bron, 2001; Mokhtarzadeh et al., 2011). Cellular hyper-fluorescence seen in dry eye disease is often presumed to be caused by increased ingress of fluorescein due to a barrier dysfunction (e.g. mucin loss, apoptosis (Bandamwar et al., 2014, 2012)). However, some details are inconsistent with this hypothesis. Caspase positive cells do not admit fluorescein (Gorbet et al., 2014) and many hyperfluorescent cells do not show apoptosis (Mokhtarzadeh et al., 2011). Therefore, an active transport exchange of monocarboxylate species that includes fluorescein has been proposed in stressed cells (Yeh et al., 2013). These observations are based on the premise that greater cellular fluorescence connotes more fluorescein. However, the discovery of quenching in normal corneal epithelium raises questions about the concentration and state of fluorescein within the abnormal cells. Many mechanisms for relatively greater fluorescence

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in abnormal cells can be posited, some of which may be independent of fluorescein concentration. Future studies will be needed to clarify the molecular basis for quenching in normal corneal epithelium and capture lifetime image decays of fluorescent epithelial cells seen in the abnormal cornea. The mechanisms involved in intracellular quenching may result in a better understanding of the phenomenon associated with dry eye disease.

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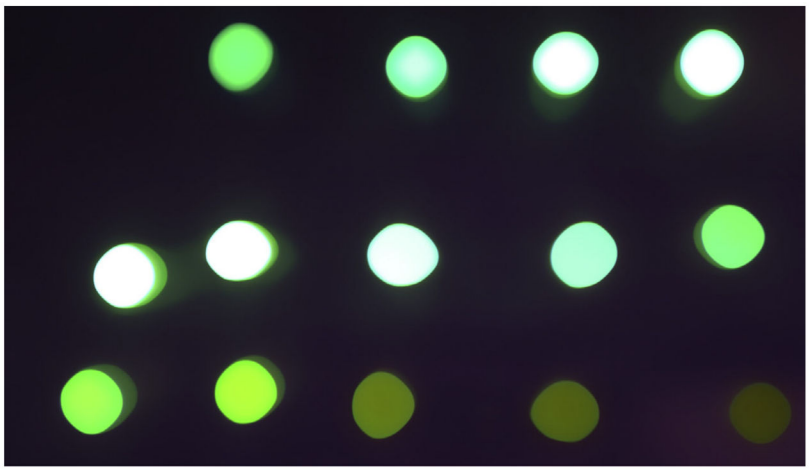

Fig. 1.

Fluorescence image from 7 μl of fluorescein spotted at increasing concentrations to show progressive quenching. Reading from left to right beginning from the top row concentrations (μM) [with the corresponding fluorescence average lifetimes (ns)] were: 203 (3.97); 492 (3.96); 1002 (3.94); 1935(3.89); 3057 (3.82); 3919 (3.72); 4890 (3.64); 6927 (3.39); 8977 (2.9); 11098 (2.881); 12388 (2.67); 15847 (1.7); 20326 (1.34); 25191 (1.12).

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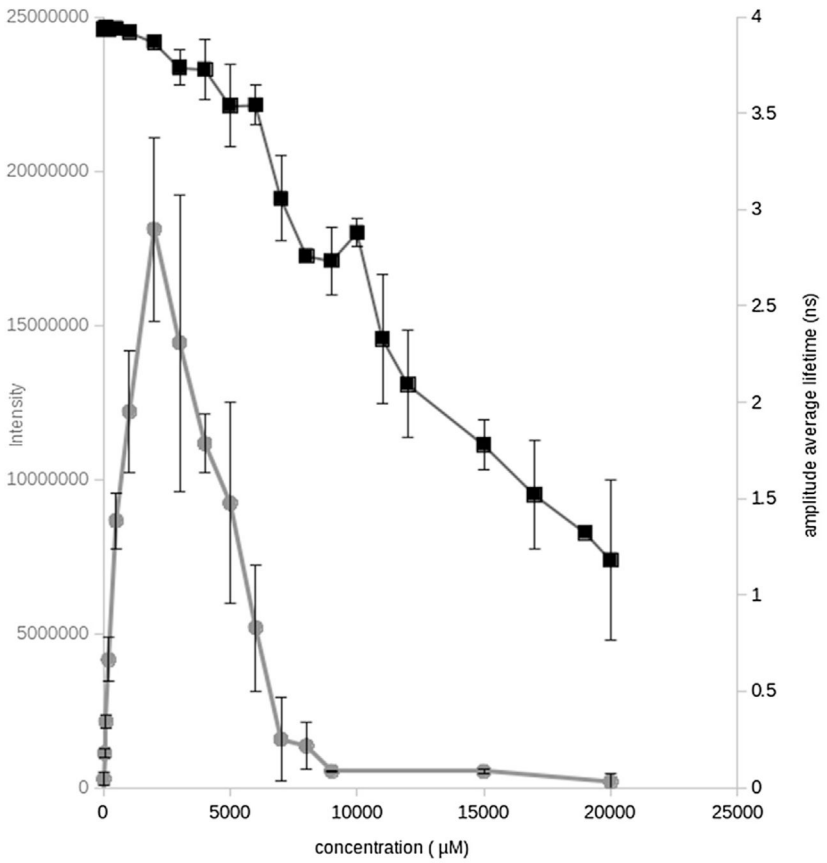

Fig. 2.

Fluorescein quenching in steady state and lifetime measurements. The means and standard deviation of binned intensities (gray circles and lines) and amplitude averaged lifetimes (black squares and lines) are shown on the y axis at various fluo-rescein concentrations. The means are taken from 3 to 6 gravimetrically measured concentrations at each point that could be binned.

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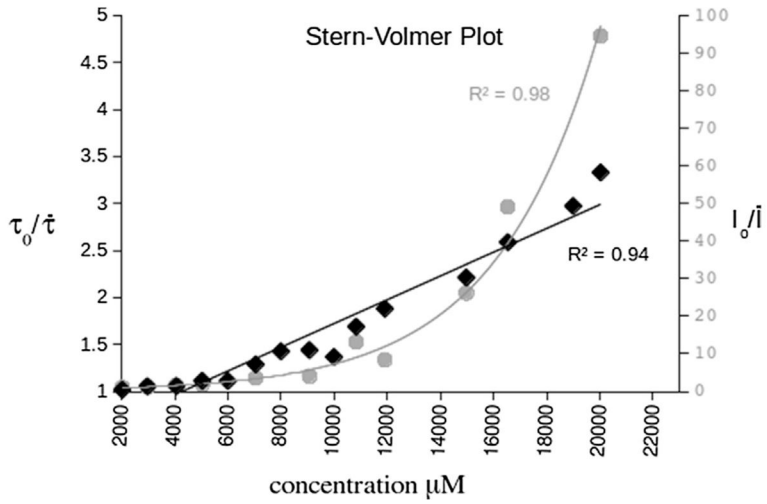

Fig. 3.

Stern Volmer Plot. The means of fluorescence intensities (gray circles and exponential fitting curve) and means of amplitude averaged lifetimes (black circles and regression line) are plotted on the primary and secondary axes respectively. Correlation coefficients are shown as R^2.

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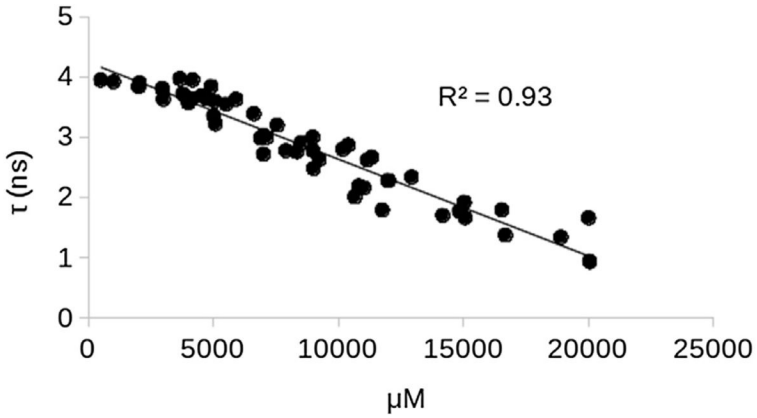

Fig. 4.

Scatter plot of amplitude averaged lifetime in nanoseconds versus concentration of fluorescein. All measurements were taken under identical instrument settings. Linear regression fitting with R^2 is shown.

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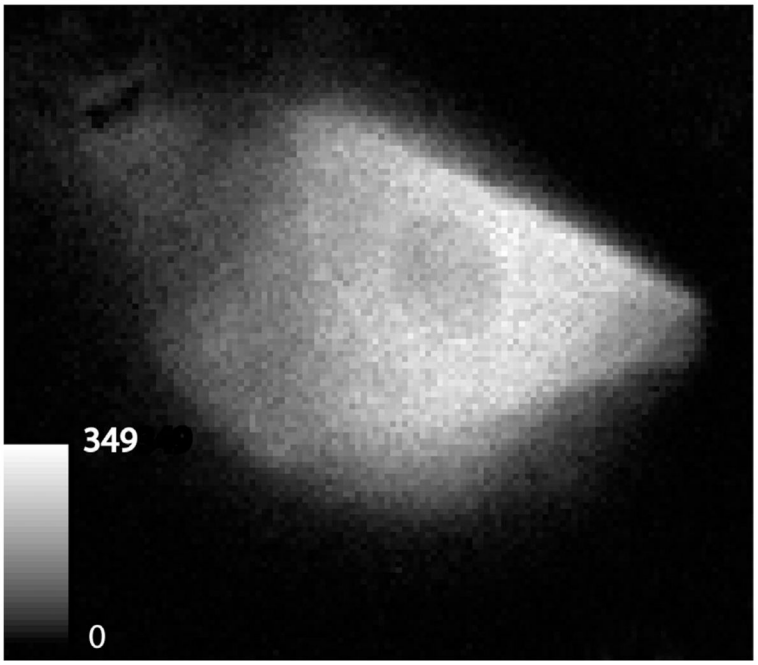

Fig. 5.

Intensity derived images of a corneal cell removed 16 min after fluorescein instillation using fluorescein 22 mM. A scale of relative fluorescence intensity is shown at the lower left.

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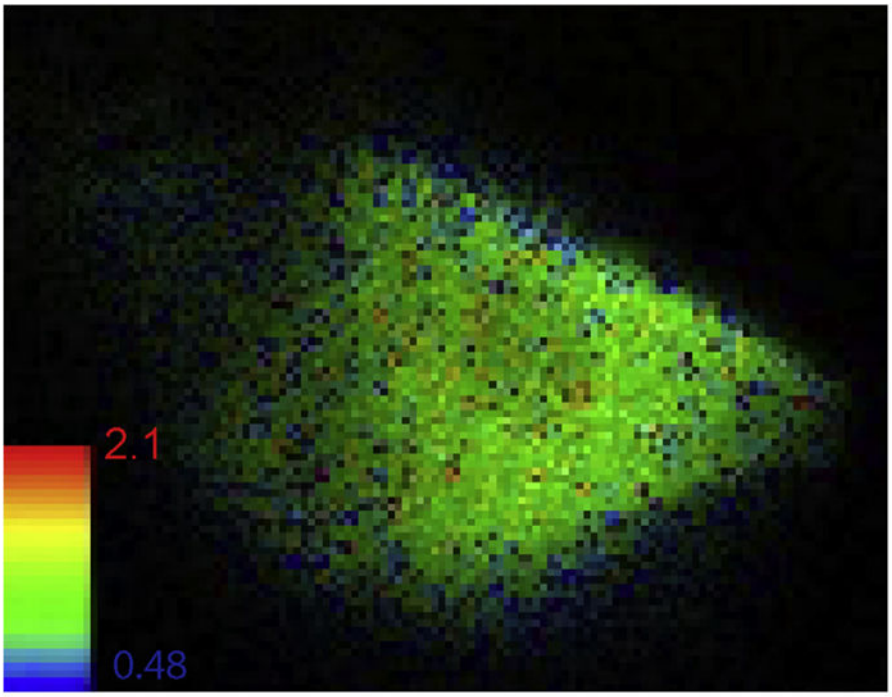

Fig. 6.

Fluorescence lifetime image of the same cell as in Fig. 5. A scale of the range of lifetimes is shown in the lower left in nanoseconds. The amplitude averaged lifetime for this image was 0.79 ns.

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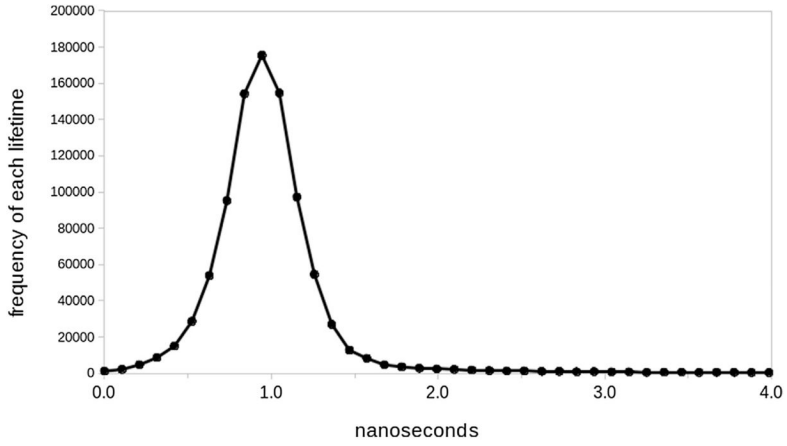

Fig. 7.

Frequency distribution of averaged fluorescence lifetimes for the cell shown in Fig. 5. The lifetime decays could not be fit to a mono-exponential model. As indicated numerous lifetime decays are represented between 0.5 and 1.5 ns indicating marked intracellular quenching by a complex mechanism.

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Table 1

Reported threshold for concentration dependent reduction of fluorescein fluorescence.

Reference	μM (% solution (grams/100 ml)	Methodology
(Umberger and La Mer, 1945)	98.4 (0.0037)	Lifetime
(Pringsheim, 1949)	452 (0.017)	Intensity
(Van Duuren, 1961)	984 (0.037)	Intensity
(Maurice, 1967)	26,596 (1)	Intensity (tear film)
(Webber and Jones, 1986)	53,192 (2)	Intensity (tear film)
(Wessing, 1969)	266 (0.01)	Intensity
(Doughty, 2010)	212 (0.008)	Intensity
(Nichols et al., 2012)	5053 (0.19)	Intensity
(Luchowski et al., 2010)	100 (0.0037)	Lifetime (PVA film)
Present study	2048/500 (0.077/.018)	Intensity/lifetime