

Two-Photon Fluorescence Microscopy of Corneal Riboflavin Absorption Through an Intact Epithelium

We write as a follow-up to our 2014 *IOVS* paper¹ describing two-photon fluorescence (TPF) microscopy of corneal riboflavin absorption. In an epithelium-off ex vivo porcine model, we reported depth-specific quantitative measurements of riboflavin concentration in the stroma. We showed that riboflavin TPF signal is significantly attenuated with increasing depth in corneal tissue. To correct this, we described a novel time-lapse measurement approach carried out during the riboflavin soak in which each cornea acts as its own internal reference. Although epithelium-off cross-linking (CXL) remains the clinical gold standard, epithelium-on CXL is heralded as delivering the benefits of standard epithelium-off treatments without the pain and complications of epithelial removal. In this letter, we address two key challenges of measuring riboflavin penetration through an intact epithelium by using our original methodology in an updated epithelium-on model.

First, we observed significant, and variable, TPF signal attenuation from the epithelium itself. We used ex vivo rabbit globes (less than 12 hours postmortem) to match human corneal epithelial thickness more closely than the porcine corneas used in our original experimentation. The Figure shows TPF signals achieved when imaging through a reservoir of riboflavin above the globe, mimicking our original setup. The TPF signals within the cornea can be seen to rise with increasing time, along with a drop in signal within the reservoir as the dye moves from the reservoir into the cornea. However, the peak tissue signal even after soaking for 2 hours is approximately one-tenth of that in the reservoir. Removing the epithelium from the same globe and immediately reimaging the underlying stroma yields an almost 4-fold increase in TPF signal (Fig., epithelium-off plot). This epithelial-masking effect was observed in all imaged eyes.

There was no consistent ratio between the signal achieved before or after epithelial removal, nor within the same eye when imaging through varying epithelial thicknesses at different locations on the test corneas.

Second, we were unable to achieve a fully soaked cornea in which the riboflavin concentration within the stroma equaled that of the reservoir, thereby compromising our attenuation-correction model. When soaking epithelium-off, we previously described the TPF signal recorded at the back of (porcine) corneas plateaued by 50 to 60 minutes, with no further increases observed with continuing soak time. This indicated the tissue was completely and uniformly soaked, with attenuation of the signal preventing a flat signal plot. This plateaued plot was used to model the signal attenuation for each eye, providing an internal depth-specific attenuation reference to correct for TPF signal loss within the stroma. However, when soaking through an intact epithelium, we were unable to achieve a steady signal at the back of the cornea. In the example shown (Fig., inset), the posterior corneal signal peaked at approximately 60 minutes, before dropping at 120 minutes. Soaking for these extended durations resulted in an approximate 10% drop in reservoir signal, as well as riboflavin moving intraocularly, with a rising TPF signal detected within the anterior chamber, confirming the presence of a third compartment for riboflavin. Despite soaking for more than 3 hours (not shown) we were unable to equalize the riboflavin concentration within the cornea to that of the reservoir. These observations significantly compromise the utility of our two-compartment attenuation-correction model, which assumes the concentration within the riboflavin reservoir above the cornea remains constant throughout the entire soak, and that in a fully soaked cornea, the riboflavin concentration within the cornea equals that of the reservoir.

We conclude that our previously described method of TPF microscopy of corneal riboflavin absorption, although suitable for epithelium-off imaging, is unsuitable when imaging through an intact corneal epithelium. Although

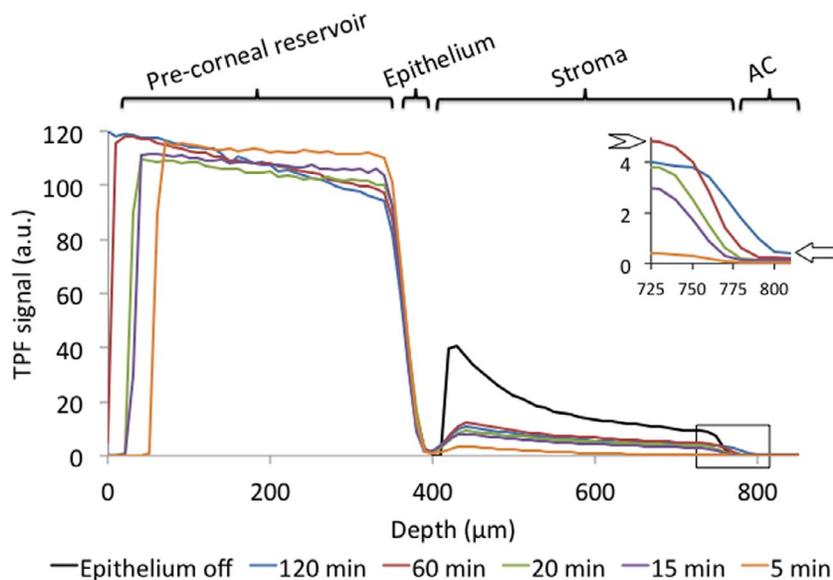


FIGURE. Two-photon fluorescence signal of ParaCel riboflavin solution (Avedro, Inc., Waltham, MA, USA) imaged through an intact rabbit epithelium. Inset magnifies the transepithelial signals at the back of the cornea revealing a peak at 60 minutes (*arrowhead*), before dropping by 120 minutes. Rising TPF signal within the anterior chamber (*arrow*) confirms the presence of a third compartment for riboflavin. AC, anterior chamber.

beyond the scope of this study, it may be possible to improve the accuracy of this technique by using a more sophisticated model of diffusion coefficients that would have to be measured in situ.

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References

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