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# Corneal Metabolic State Assessment by Fluorescence Lifetime Imaging Microscopy

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A long time objective of ophthalmologists is to diagnose corneal cells dysfunction prior to its pathologic expression. With this motivation, we are currently developing a new instrument for in vivo metabolic imaging of corneal tissues.

Metabolic alterations are known to be the first sign of several corneal pathologies and can be assessed through non-invasive monitoring of metabolic co-factors flavin adenine dinucleotide (FAD) and nicotinamide adenine dinucleotide (NADH). The quantification of the relative proportions between free and protein-bound NADH and FAD can be achieved using fluorescence lifetime-resolved methods. This approach has already been applied in age-related macular degeneration [1], diabetic retinopathy [2] and epithelial cancer [3].

FAD and NADH imaging can be performed by one-photon excitation (1PE) and two-photon excitation (2PE) techniques. The latest has the advantage of allowing simultaneous excitation of both metabolic co-factors. However, there are still safety concerns when considering in vivo ocular studies in humans using 2PE.

Due to these concerns we used, as a first approach, a 1PE system for evaluating the feasibility of corneal FAD imaging. The use of FAD has advantages over NADH. It can be excited over longer excitation wavelengths, is more resistant to photo-bleaching and is located exclusively in the mitochondrial space.

A PicoQuant MicroTime 100 (PicoQuant GmbH, Berlin, Germany) coupled to an Olympus BX51 Microscope (Olympus Corporation, Tokyo, Japan) was used to monitor FAD autofluorescence. The instrument uses a 440 nm pulsed diode laser (330 ps) running at a pulse rate of 40 MHz. The instrument was modified by us to allow the acquisition of both fluorescence lifetime and reflectance images and to enhance scattered light rejection.

Intensity decay curves were processed with SymPhoTime v5.3 Software (PicoQuant GmbH, Berlin, Germany). The fluorescence decay times were obtained after applying a non-linear least square fit to the decay data and the goodness of fit was evaluated by the analysis of the residuals and the chi-squared ($\chi 2$).

We have acquired fluorescence lifetime images of ex vivo healthy Wistar rat corneas (Fig.1) using two different instrument setups: 1- using the emission filters provided by the manufacturer; 2- placing extra emission filters to fully reject the scattered excitation light. In both setups, FAD fluorescence data presented a bi-exponential decay with a short (protein-bound FAD) and a longer (free FAD) lifetime component.

While both setups provide FAD fluorescence decays, only the second retrieves valid metabolic information. We obtained two lifetime components, one of 0.118 (0.028) ns and another of 2.11 (0.16) ns, with a relative contributions of 39.4 (2.2) and 60.6 (2.2), respectively. These values are in accordance with the literature [3,4].

Corneal layer discrimination is possible based on morphologic characteristics. However, the fluorescence lifetime images do not provide morphological detail (Fig.1), possibly because FAD is only present in the mitochondria. These organelles are small and tend to accumulate around the nuclei.

So, we modified the instrument's optical setup to allow the acquisition of both fluorescence lifetime images and reflectance images. Figure 2 shows an example of the corneal epithelial layer.

The image resolution and depth penetration are still not ideal. Since the assessment of corneal endothelial layer metabolic function is also within our goals, we are currently implementing further modifications to improve both the instrument's resolution and depth penetration.

The characterization of FAD fluorescence lifetime in unhealthy corneas is important to detect corneal dysfunctions prior to its pathologic expression. Therefore, we intend to study metabolic altered Wistar rat corneas. The alterations will be induced by potassium cyanide, which is a reversible inhibitor of the fourth complex of the mitochondrial electron transport chain.

References

1. Schweitzer D. *et al*., Proc. of SPIE., 4432:29-39, 2001.
2. Schweitzer D. *et al*., Proc. of SPIE-OSA Biomedical Optics, 7368:736802, 2009.
3. Skala M.C. *et al*., PNAS, 104:19494-19499, 2007.
4. Nakashima N. *et al*., J. Biol. Chem., 255:5261-5263, 1980.

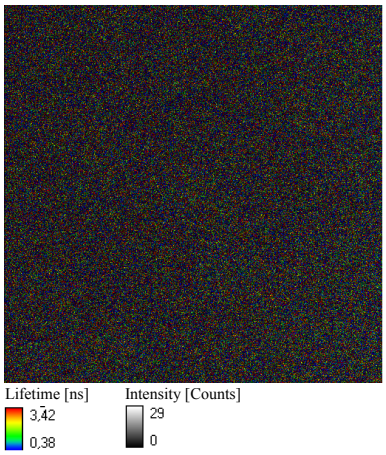


**Fig1.** Fluorescence lifetime image of *Wistar* rat cornea.

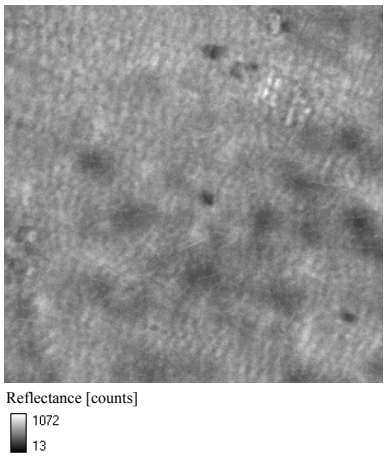


**Fig2.** Reflectance image of the corneal epithelium of *Wistar* rat.

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