# Summary of the doctoral dissertation

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# Application of two-photon excitation to study early changes in the retina associated with environmental stress, genetic manipulations and drug treatment

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**Study objectives**:

1. Develop a two-photon fluorescence-based, noninvasive methodology for characterizing changes in retinal structure and function in healthy and disease-affected retina.
2. Develop a two-photon fluorescence-based assay to identify the sequence of events leading to retinal degeneration and characterize the time line of events leading to retinal pathology after induction of stress.
3. Characterize the impact of pharmaceuticals on the fluorescent signature of the earliest signs preceding photoreceptor cell death and identify the components of the pathological deposits associated with retinal degeneration by two-photon fluorescence-based retinal imaging.

**Theses of the dissertation:**

1. Structural changes in the retina and retinal pigment epithelium (RPE) leading to photoreceptor death could be identified within 24 hours after stress onset.
2. Modulation of retinoid cycle enzymes either genetically or pharmaceutically can prevent or exacerbate retinal degeneration.
3. Two-photon imaging integrated with fluorescence spectral measurements can provide information about the detailed composition and fluorescent fingerprints of different types of retinal deposits.

**Conducted research and the obtained results:**

Results of performed studies were published in 6 scientific articles. The 6 articles were published in the journals from ISI Master Journal List.

To validate two-photon fluorescence (TPF) imaging for monitoring visual (retinoid) cycle in humans, TPF images of the retina and the content of two types of retinoids participating in the visual cycle were measured in primate eyes [1]. In this study, the analysis of human retinal extracts by high-performance liquid chromatography revealed that the sum of both *cis* and *trans* isomers was on average ~4 pmol per $mm^2$ of retinal area. This compares well with previous reports of retinyl esters in the retinas of wild type (WT) mice [55]. Moreover, in this study the average amount of A2E, a major condensation product of all-*trans* retinal, in the primate eye was ~0.2 pmol per $mm^2$ of retinal area, comparable with a previously reported value of ~0.3 pmol per $mm^2$ of retinal area in 6-month-old WT mice. These observations indicate that TPF imaging is informative for retinal imaging not only in mice but also in monkeys and ultimately humans to enable the early evaluation of genetic, environmental and therapeutic factors on the health of the retina.

To evaluate the retina in live mice, a TPF imaging instrument containing a Ti:Saphire laser with integrated group delay dispersion pre–compensation and a sensor-less adaptive optics (AO) system was assembled [2]. Briefly, laser light was modulated with a deformable mirror (DM) and routed into an upright microscope equipped with galvanometric scanners, a custom objective with 0.5 NA, and a photomultiplier tube detector in a non–descanned configuration. Initial images of the retinal pigment epithelium (RPE) created by endogenous fluorophores were obtained with *ex vivo* mouse eyes submerged in phosphate–buffered saline (PBS). Optimization of the dispersion pre-compensation increased the mean fluorescence an average of 5–fold relative to conditions without pre–compensation. Optimization of the DM, based on a feedback signal from the detector, had a positive impact on both mean fluorescence which increased from 34.6 to 58.1 arbitrary units and on the dynamic range of the images quantified as the range of pixel values which increased from 176 to 237 arbitrary units with 255 as the maximum. Experiments in live pigmented $Abca4^{-/-}Rdh8^{-/-}$ mice demonstrated that the fluorescent granules, that showed up in the retina of these mice after exposure to bright light, were responding to 850 nm excitation and were located 3.0 mm away from the cornea. Such granules were not detected in animals treated with retinylamine before exposure to bright light. Retinyl ester storage compartments, retinosomes, in live $Rpe65^{-/-}$ mice were successfully visualized 3.2 mm posterior to the cornea with 730 nm excitation light. Moreover, the two-photon excited fluorescence emission spectra revealed the presence of distinctly different fluorophores in the RPE of $Rpe65^{-/-}$ and $Abca4^{-/-}Rdh8^{-/-}$ mice.

Furthermore, to image the back of the eye in larger animals, a custom periscope objective was designed and incorporated in the TPF with AO set up [3]. The newly designed periscope for mice eliminated the need for a thick, eye flattening contact lens and provided proof of concept of the design for application in humans. As a further benefit, images of the RPE and their subcellular organelles, retinosomes, in WT and *Rpe65*$^{-/-}$ mice, obtained with this periscope objective had a larger field of view than could be obtained with the simpler 0.5 NA objective [3].

TPF imaging revealed distinct features of the RPE in intact eyes of mice carrying a double knockout (DKO) of *RDH8,* the enzyme that reduces all-*trans*-retinal to all-*trans*-retinol, and the *ABCA4*, the transporter responsible for the translocation of all-*trans*-retinal (atRAL) from the inside to the outside of the photoreceptor disc membranes [4]. Two-photon excited emission spectra from WT and DKO mice RPE clearly differed. With 730 nm excitation light, the emission from WT mouse RPE had a maximum around 480 nm, consistent with the two-photon spectrum of all-*trans*-retinol and/or all-*trans*-retinyl esters. In contrast the DKO mouse retina displayed maximum emission at 565 nm, indicative of the over-accumulation of retinoid condensation products, such as A2E, consistent with the expected DKO phenotype. With 910 nm excitation light, not only did the emission maximum shift to longer wavelengths in both types of mice, indicating a mixture of different fluorophores, but the emission spectra from WT and DKO mice RPE also overlapped indicating that fluorescence from condensation products dominates in mature WT mice. Moreover, the ratios of fluorescence generated by 910 nm to that generated by 730 nm light systematically increased with age in WT mice and were more than double in age-matched DKO mice, confirming that aging WT mice accumulate condensation products though at a slower pace than DKO mice. These imaging results were validated by measurements of the retinoids in the eyes of WT, DKO and *Rpe65*$^{-/-}$ mice. Retinoid contents were consistent with the TPF excitation data [4].

Identifying the sequence of events underlying light induced pathology is important for delineating the mechanisms leading to retinal degeneration, and consequently for the development of therapies against retinal diseases [5]. To determine the timeline of degenerative processes in the retina initiated by bright light exposure, the *Abca4*$^{-/-}$*Rdh8*$^{-/-}$ mouse model was selected since it exhibits many features associated with human Stargardt disease and age-related macular degeneration. TPF imaging demonstrated that rod photoreceptors are the initial loci of light induced retinal degeneration. The first changes were observed by 24 hours following exposure to bright light. Primary changes included retinoid-

dependent formation of fluorescent metabolic by-products within rod photoreceptor cells, a nearly three−fold expansion/swelling of the cells. These changes were followed by secondary infiltration of microglia/macrophages to clear photoreceptor cell debris, and subsequent formation of long-wavelength fluorescent granules in the RPE. These changes were concomitant with the loss of function as evidenced by electroretinography measurements [5].

TPF evaluation after bright light exposure also was used to assess the protective impact of treatments with apocynin, emixustat, retinylamine and MB-002 [5, 6]. Apocynin is an NADPH oxidase inhibitor. Emixustat, retinylamine and MB-002 are similar in structure and are inhibitors of RPE65, but MB-002 does not have an amine group, which is critical for trapping excess all-*trans*-retinal. Apocynin, retinylamine and emixustat fully protected the photoreceptors from light-induced degeneration, whereas the protection by MB-002 was marginal. Moreover, to confirm that differences in the protective effects of emixustat versus MB-002 against light induced retinal degeneration are not limited to mice lacking ABCA4 and RDH8, the impact of these compounds on retinal preservation in BALB/c mice was compared. Treatment with emixustat fully protected the retinas of these mice from light-induced damage, whereas protection conferred by treatment with MB-002 again was marginal. Although twice the light exposure (both intensity and duration) was used to treat BALB/c mice as compared to the exposure used for *Abca4*$^{-/-}$*Rdh8*$^{-/-}$ mice, the number of damaged photoreceptors was less in the BALB/c mice, indicating greater resistance of WT mouse retina to environmental insults. The greater ability to protect retina from light induced damage by atRal trapping pharmaceuticals than by the inhibitor of the RPE65 without an amine group demonstrates that light-induced retinal degeneration in these species is likely induced by atRAL released from visual pigments after the initial photobleach rather than from visual pigments regenerated during the light exposure [6].

**Conclusions**

In the course of this research, the TPF imaging system and methods for studying biochemical processes in retinas affected by genetic disorders, environmental stress or pharmaceutical treatments were developed. TPF imaging and the measurement of retinoid content in primate and mouse eyes indicate that a two-photon excitation-based technique is effective for retinal imaging not only in mice but also in monkeys and ultimately humans. Differences in the content of intermediates and condensation products (byproducts) of the retinoid cycle in mice at different ages and genetic backgrounds were demonstrated with the use of TPF and validated by analytical measurements of retinoid content. Furthermore, the

newly developed technique, for the first time, enabled spectral characterization and acquisition of the images of retinoid cycle fluorophores in the RPE of living pigmented mammals. Applying TPF to study the timeline of retinal changes after exposure to bright light resulted in the discovery that rod photoreceptors are the initial loci of retinal degeneration. The first changes were observed by 24 hours following exposure to bright light. Finally, these studies demonstrated that the onset of retinal degeneration could be prevented by treatments with apocynin, emixustat and retinylamine and to a lesser extent MB-002.

Two-photon fluorescence excitation imaging is a powerful method for studying the earliest changes in the retina and RPE, and for detecting early abnormalities in the structure of the retina as well as the efficiency of biochemical processes fundamental to sustaining vision. These capabilities are crucial for the development and screening of drug candidates and individualized therapeutic modalities aimed at saving vision.

**Publications that are the basis of applying for doctoral degree:**


1. **Palczewska G**, Golczak M, Williams DR, Hunter JJ, Palczewski K; "Endogenous fluorophores enable two-photon imaging of the primate eye". Investigative Ophthalmology & Visual Science, 2014 Jun 26;55(7):4438-47.
   **IF = 3.404, MNiSW = 40**, wykaz A, pozycja 5321, nr ISSN: 0146-0404

2. **Palczewska G**, Dong Z, Golczak M, Hunter JJ, Williams DR, Alexander NS, Palczewski K; "Noninvasive two-photon microscopy imaging of mouse retina and retinal pigment epithelium through the pupil of the eye". Nature Medicine, 2014 Jul;20(7):785-9.
   **IF = 27.363, MNiSW = 50**, wykaz A, pozycja 8267, nr ISSN: 1078-8956

3. Stremplewski, P., K. Komar, K. Palczewski, M. Wojtkowski, **G. Palczewska**. "A periscope for noninvasive two-photon imaging of murine retina in vivo". Biomedical Optics Express, 2015 Aug 13;6(9):3352-61
   **IF = 3.648, MNiSW = 40**, wykaz A, pozycja 1444, nr ISSN: 2156-7085

4. **Palczewska G**, Maeda T, Imanishi Y, Sun W, Chen Y, Williams DR, Piston DW, Maeda A, Palczewski K.; "Noninvasive multiphoton fluorescence microscopy resolves retinol and retinal condensation products in mouse eyes". Nature Medicine; 2010 Dec;16(12):1444-9.
   **IF = 27.363, MNiSW = 50**, wykaz A, pozycja 8267, nr ISSN: 1078-8956

5. Maeda A*, **Palczewska G***, Golczak M, Kohno H, Dong Z, Maeda T, Palczewski K; "Two-photon microscopy reveals early rod photoreceptor cell damage in light-exposed

mutant mice". Proceedings of the National Academy of Sciences of the United States of America, 2014 Apr 8;111(14):E1428-37.
**IF = 9.674, MNiSW = 45**, wykaz A, pozycja 9267, nr ISSN: 0027-8424

6. Zhang J, Kiser PD, Badiee M, **Palczewska G**, Dong Z, Golczak M, Tochtrop GP, Palczewski K; "Molecular pharmacodynamics of emixustat in protection against retinal degeneration". The Journal of Clinical Investigation, 2015 Jul 1;125(7):2781-94.
**IF = 13.261, MNiSW = 50**, wykaz A, pozycja 5812, nr ISSN: 0021-9738

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