2:00 p.m.	D. Schweitzer (DE-Jena)

Key Note Lecture:
Fluorescence lifetime imaging of the living human retina
Purpose: To find a method for the evaluation of cellular metabolic state at the fundus. Methods: Analysing specific conditions for FLIM application, a fluorescence lifetime laser scanner ophthalmoscope was developed based on a modified Heidelberg Retina Angiograph. According to measurements on expected pure fluorophores and isolated ocular structures, the auto-fluorescence can be excited at 2 different wavelengths (448 nm, 468 nm) and detected by TCSPC technique in 2 spectral ranges (CH1=490-560 nm, CH2=560-00nm). Eye movements are compensated by online image registration. The time-resolved fluorescence intensity is approximated by a tri-exponential model function. A fit algorithm was developed which considers the layered structure of the eye. In ongoing studies lifetime measurements were performed on healthy subjects, age-related macular degeneration, retinal vessel occlusion, and diabetes mellitus. Local metabolic alterations can be demonstrated in images of lifetime, amplitudes, or relative contribution. Global changes can be found by statistical analysis in histograms of fit parameters. Results: Comparing with the fundus anatomy, the shortest component ($T1 \approx 110$ ps, $a1 \approx 87\%$) corresponds with retinal pigment epithelium and the middle component ($T2 \approx 500$ps, $a2 \approx 10\%$) with neuronal retina. The longest component ($T3 \approx 1.5$-5 ns) is influenced by the fluorescence of the lens. Significant differences were found between healthy subjects and early AMD in T1 (118 ± 23ps vs. 166 ± 53ps) and in T2 (584 ± 184ps vs. 968 ± 408ps) of CH1. In diabetes, all lifetimes are elongated in comparison with normals. Conclusions: FLIM opens a new way for detection of early pathologic changes when alterations are reversible. But the interpretation of the detected changes requires the co-operation of physicians, biochemists, and physicists.

2:50 p.m.	S. Jentsch (DE-Jena)

Characterization of ocular tissues measured by fluorescence and excitation spectra as well as fluorescence lifetime imaging
Purpose: The aim of this study was the characterization and differentiation of ocular tissues measured by fluorescence spectra and lifetimes as well as the comparison with fluorescence parameter of pure endogenous fluorphores. Method: The ocular tissues cornea, lens, vitreous, neuronal retina, pigment epithelium, choroid, sclera and aqueous humour were separated from porcine eyes about one hour post mortem. The measurements of excitation and emission spectra were performed using the spectrometer LS 5 (Perkin Elmer). The spectra were averaged over measurements in tissues form 10. The fluorescence lifetimes were measured with a modified Scanning- Laser- Ophthalmoscope (Zeiss, Oberkochen) for picosecond excitation at 446 nm and 468 nm and determined applying the program SPCImage 2.7 (Becker/Hickl) with a bi-exponential approximation. The fluorescence lifetime parameters Ti (lifetime), Ai (amplitude) and Qi (relative contribution) were determined. Results: The lens showed the highest fluorescence intensity. The spectral characteristics of

the ocular tissues is determined by the fluorophores NADH, FAD, collagenes, elastin, tryptophan, protoporphyrin IX, and others having partially overlapping emission spectra. NADH was probably detectable in all structures with emission maximum at 460 nm. In the same way a maximum at 530 nm was detected in all structures, which is specific for FAD. Whereas the emission spectra of the ocular tissues were similar, a discrimination was obtained by measuring the fluorescence lifetimes. The retinal pigment epithelium (RPE) and the neuronal retina significantly differ from each other considering the relative contribution Q1. The vitreous also differs from nearly all other ocular structures. Similar fluorescence lifetimes were detected in sclera, cornea and lens. Conclusion: The in-vivo fluorescence lifetime imaging (FLIM) of a human retina could be better interpreted with the acknowledgement of fluorescence parameters of each single ocular tissue.

3:15 p.m.	L. Deutsch (DE-Jena)

Ophthalmic FLIM for discrimination of diabetics and healthy subjects
Purpose: Applying ophthalmic FLIM for diagnostics of early diabetic alterations in the eye. Method: Diabetes mellitus is a systemic metabolic disease. In advanced stages, untreated diabetic alterations can lead to blindness. To choose treatment in time, the detection of earliest metabolic changes in the eye is necessary. The autofluorescence of endogenous fluorophores which characterizes the metabolic state can be detected by FLIM. FLIM measurements are performed on 93 diabetic and 34 healthy subjects. Three-exponential fit is applied for photon histograms in a short-wavelegth channel Ch1:490-560 nm and in a long-wave channel Ch2 560-700 nm. In a first statistical analysis, parameters are compared which characterize the individual distribution of amplitudes, lifetimes, and relative contributions in both spectral channels. In a second comparison, sum histograms of amplitudes, lifetimes, and relative contribution for all healthy subjects and for diabetic patients are calculated according to the Holm method. Individual local alterations - especially regions of reduced metabolism - are detectable in images of the fit parameters. Results: Whereas no significant differences were found for the amplitudes, lifetimes and relative contributions are different between healthy subjects and diabetic patients. FLIM parameters are more different in CH1 than in Ch2. High significant alterations were found for T2 and T3 in Ch1 but not in T1. Conclusion: As T2 originates in the neuronal retina, diabetic alterations occur in this anatomical layer, but not in the RPE where T1 originates. Elongation of T3 is determined by accumulation of advanced glycation end-products.

3:40 – 4:00 p.m. Coffee break

4:00 p.m.	M. Klemm (DE-Ilmenau)

Reproducibility of Ophthalmic Lifetime Mapper measurements

Purpose: To determine the reproducibility of fluorescence lifetime imaging (FLIM) measurements in ophthalmology on the basis of an evaluation of fluorescence decay photon histograms and by means of multi-exponential component fitting of detected photon distributions. Methods: For this study a Heidelberg Retina Angiograph was modified to measure the fluorescence lifetime at the human retina. This fluorescence lifetime mapper contains a diode laser which emits 446 nm pico-second pulses at 80 MHz repetition rate. The detection of auto-fluorescence was done in wavelength channel 1 at 490-560 nm and channel 2 at 560-700 nm by time-correlated single photon counting. The laser pulse interval was divided into 1024 time channels of 12.2 ps each. A lateral resolution of 40 x 40 m^2 was achieved. For this study ten healthy male subjects aged between 25 and 35 years were examined. Each subject was measured twice per day (with one hour break in-between) on two days a week over three weeks resulting in a total of ten datasets per subject. Three regions (fovea centralis; a blood vessel free region of the papilla-macula-bundle; optic disc) each with a size of 15 x 15 pixels were manually selected and binned into a single fluorescence decay photon histogram. In those histograms the offset (background and detector dark current) was subtracted, the pre-stimulus part was removed and the maxima were normalized. A two-sample Kolmogorov-Smirnov (KS) test was performed for all unique permutations of the ten repetitive measurements separately for each spectral channel at a 5 % significance level. The decay of the fluorescence emission was approximated by a tri-exponential model function to determine the amplitudes a1, a2, a3 and lifetimes t1, t2, t3 in both wavelength channels. Results: The KS test shows that the measured photon distributions are significantly different in the short wavelength channel for about 6 % and in the long wavelength channel for about 8 % over all regions and subjects. The largest differences occur in the optic disc region (12 % on average) due to the generally low photon counts while the smallest differences occur in the macula region (1 % on average). Comparing both spectral channels with the KS test states in about 95 % of the cases different distributions proving that there is different information in both channels. The comparison of the first and the second measurement per day did not reveal significant differences. Mean values for amplitudes are a1=86.6%, a2=10.1%, a3=3.3% and for lifetimes t1=106 ps, t2=581 ps, t3=2218 ps in the papilla-macula bundle for channel 1 and a1=79.7%, a2=16.5%, a3=3.8% and for lifetimes t1=121 ps, t2=474 ps , t3=1488 ps for channel 2. The intra-individual reproducibility was different between the subjects. The best reproducibility (<1%) in all regions was detected for amplitude a1 and the worst in most locations for a3 (up to 30%). The typical coefficient of variation was less than 10% for the lifetimes. Because of the low number of photons, the reproducibility of lifetimes in the optic disc can be up to 37%. Conclusions: The reproducibility of this new method for functional diagnostics of metabolic changes allows for the reliable application of subsequent analysis and diagnosis methods. The amplitudes achieve most precise but less stable values in contrast to the lifetimes which are more stable by considerable accuracy.

4:25 p.m.	A. Dietzel (DE-Ilmenau)

Co-registration of FLIM and OCT measurements of the human eye

Purpose: The aim is to combine information between cross-sectional images (OCT) and metabolic data (FLIM).

Methods: The FLIM measurement at the human fundus was accomplished by a modified Heidelberg Retina Angiograph. Two spectral channels were used for auto-fluorescence detection with application of time-correlated single photon counting. A 30° region of the fundus was covered with a lateral resolution of 40 x 40 μm^2 resulting in one fluorescence intensity (FI) image per channel (150 x 150 pixel, up to several 1.000 gray scales). The cross-sectional images of the retina were acquired by a Heidelberg Spectralis OCT. For each scan an infrared image (1536 x 1536 pixel, 256 gray scales) was used to compensate eye-movements which covers a 30° area at the fundus. It was saved along with the cross-sectional scans and their positions on the infrared image. In the framework of a reproducibility study, which included ten healthy male subjects (age 25 to 35), each subject was measured ten times at the fluorescence lifetime mapper and once at the Spectralis OCT. Since the nature of both image data is very different and a non-rigid transformation is required, a particle swarm optimizer with different distance measures was designed. One was Mutual Information which is considered as the state-of the-art distance measure and the other is known as Normal Gradient Fields. Furthermore a multiresolution approach was implemented to ensure a low number of convergence cycles.

Results: Experimental results with Mutual Information as the distance measure showed that an good level of accuracy can be achieved to register OCT images and FI images. In case of Normal Gradient Fields as the distance measure, the results showed to be more accurate. Attention should be paid to the quality of the FI images. Doubled retinal vessels caused by eye- and head-movements led to bad coregistration results in both cases.

Conclusions: The demonstrated coregistration of functional and structural data might improve future diagnostic approaches.

4:50 p.m.	M. Kaatz (DE-Saarbrücken)

In vivo diagnosis of malignant melanoma by multiphoton laser tomography

Aims: The incidence of malignant melanoma has shown a dramatic increase over the past three decades. On the one hand, patient outcome and curability depend on early diagnosis, on the other hand, only few excised melanocytic skin lesions turn out to be malignant at histopathological examination. Therefore, non-invasive diagnostic of pigmented skin lesions is of outstanding interest. In vivo multiphoton laser tomography (MLT) represents a recently developed diagnostic tool that allows non-invasive tissue imaging. Methods: In the investigation of eighty-three melanocytic skin lesions by MLT we identified distinct morphological differences in melanoma compared with melanocytic nevi. In particular, six characteristic features of malignant melanoma were specified and statistically evaluated. Furthermore, we investigated fluorescence intensity and lifetime in order to yield additional information for diagnostics of suspicious pigmented skin lesions. Results: Sensitivity values up to 95% and specificity values up to 97% were achieved for diagnostic classification. The most significant diagnostic criteria include architectural disarray of the epidermis, poorly defined keratino-

cyte cell borders as well as the presence of pleomorphic or dendritic cells. Remarkable differences in lifetime behaviour of keratinocytes in contrast to melanocytes were detected. Fluorescence lifetime distribution was found to correlate to the intracellular amount of melanin. Excitation at 800 nm shows a selectively observable fluorescence of melanin containing cells and offers the possibility of cell classification. Conclusion: Procedures of selective imaging as well as spectral fluorescence lifetime imaging by means of multiphoton laser tomography support diagnostic decisions and may improve the process of non-invasive early detection of melanoma.

End of Lecture Session