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Combining LAURDAN Generalized Polarization, Fluorescence Correlation Spectroscopy and Fluorescence Lifetime Imaging as a Tool in Skin Diagnostics

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The outermost layer of the skin tissue, i.e. the stratum corneum (SC), plays a key role in the barrier function of the skin. SC (10 to 20 micrometer thick) is comprised of dead, keratin filled cells residing in a matrix of complex extracellular lipids. Physical related features of skin SC such as the lipid lateral packing in the extracellular matrix, local proton (water) activity, or local diffusion of particular substances are crucial in determining the permeability of skin towards exogenously added compounds (e.g. drugs and cosmetics). In addition, these parameters may be altered in abnormal skin and further on used as biomarkers for detecting skin anomalies. In this work we decided to map and compare proton activity, polarity (that in turn is related to lipid packing of skin SC membranes) and local diffusion of particular substances in excised skin samples, obtained from wild type mice and abnormal mice, showing increased transepidermal water loss. The experimental techniques utilized are based in multiphoton excitation microscopy techniques. LAURDAN Generalized Polarization (GP) measurements are used to evaluate the overall polarity of potential transepidermal pathways (1). Our results show markedly higher GP values in skin lipid membranes from mice with increased transepidermal water loss compared to controls. To further assess the properties of the skin lipid membranes at the two different physiological conditions, local diffusion through the barrier is evaluated by fluorescence correlation spectroscopy. The data obtained are related to the proton gradient across the SC, as observed by fluorescent lifetime imaging (2). This combined strategy is being tested for potential implementations in skin diagnostics.

1) D.C. Carrer, et al, Journal of Controlled Release (2008), doi:10.1016/j.jconrel.2008.08.006.
2) Hanson et al. 2002, Biophys. J. 83, pp. 1682-1690.

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Selective Detection of NAD(P)H-Dependent Enzymes during Their Function within Vital Cells by means of Time-Resolved TPLSM

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The investigation of physiological phenomena at molecular level within vital cells, for instance metabolic processes, represents a major challenge in life sciences. In this context, measuring methods should be non-invasive for the system under study and should provide high sensitivity and specificity. We demonstrate inhere the power of marker-free, spatially and timely resolved intracellular detection of NAD(P)H-dependent enzymes in the study of phenomena of particular biomedical relevance like phagocytosis or autoimmune reactions. The technique is based on biexponential two-photon laser scanning fluorescence lifetime microscopy (TPLSM FLIM) of the physiologic fluorophore NAD(P)H, which exhibits a fluorescence lifetime τ that is strongly dependent on its chemical environment, i.e. on the enzyme to which NAD(P)H is bound to (free NAD(P)H: $\tau \sim 400$ ps, enzyme-bound NAD(P)H: $\tau \sim 2$ ns). In order to verify the feasibility of the method, an in-vitro study with different enzymes in solution was performed. The measured fluorescence lifetimes of enzyme-bound NAD(P)H strongly varied from $\tau = 960 \pm 27$ ps for malic dehydrogenase to $\tau = 3640 \pm 101$ ps for 3α hydroxysteroid dehydrogenase, which made us confident, that an intracellular selective enzyme detection is possible by means of NAD(P)H-FLIM. We indeed succeeded for the first time to specifically and intracellularly detect the NADPH oxidase, a multi-subunit membrane-bound enzyme complex that catalyzes the reduction of free oxygen to its superoxide anion. Thereby, we selectively monitored the NADPH oxidase during its function within differently activated murine polymorphonuclear leucocytes (PMNs). The experiments revealed a specific fluorescence lifetime of 3670 ± 140 ps for NADPH bound to this enzyme both in humorally activated PMNs, i.e. activation with PMA, and in PMNs phagocytosing the fungus Aspergillus fumigatus, confirming the assembly of NADPH oxidase as highly site-specific.

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Imaging Collagen for Normal and Pathological Skin Dermis through Polarization-Resolved Second Harmonic Generation

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Polarization-resolved, second harmonic generation (P-SHG) microscopy is utilized for medical diagnosis of pathological skin dermis. We acquired the pitch angle of collagen fiber from the P-SHG microscopy. In analyzing the large area, pixel by pixel of normal and pathological skin dermis, we found that excessive proliferation of collagen fiber (EPC) and dissolution of elastic fiber (DEF) characterize pathological skin dermis. In addition, the pitch angle of normal skin was found to be 52.01 ± 0.96, while pathological skin EPC and DEF were found to be 48.68 ± 1.49 and 48.09 ± 1.83. It is found that pitch angle of pathological dermis trend to smaller angle, below 50 degree, and wider distribution, 2 times over the normal.

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Intravital Multiphoton Microscopy For Imaging Hepatobiliary Function In Vivo

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When liver can not be regenerated in time to amend damages that has occurred, failure of hepatic functions such as liver failure and metabolic disease can result. Traditionally, the study of liver pathology has depended on histological techniques, but such methods are limited to ex-vivo observation. In order to study hepatic metabolism in vivo, we have designed a hepatic imaging chamber made of biocompatible titanium alloy (6V4Al-Ti). In combination with multiphoton and a method of quantification by using optical signal, our approach allows the intravital observation of hepatic activities to be achieved. Processes such as hepatic metabolism and disease progression can be studied using this methodology.

1502-Pos Board B346

Second Harmonic Generation Microscopy Characterization of Corneal Edema

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The purpose of this study is to investigate the structural features of corneal edema by multiphoton fluorescence and second harmonic generation (SHG) microscopy and the potential of this technique as a clinical, *in vivo* monitoring technique for cornea disease diagnosis. Corneal edema is a build up of fluid or swelling of the cornea that can produce clouding of the cornea and significant decrease in vision. Since collagen can be induced to generate strong second harmonic generation (SHG) signal, multiphoton excitation provide direct visualization of collagen orientation within corneal stroma.

In this study, normal and over-hydrated bovine corneal specimens used in this study were observed to distinguish the structural alteration due to edema. We collected forward and backward SHG signals simultaneously within different depths and also with large region images. From the result, we found that the organization of corneal stroma was not significantly altered, except for the increase of interfibrillar spacing. This structural information provided by multiphoton imaging may help the evaluation of the necessity for full-thickness corneal transplantation in pathological cases.

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New Dyes With Fast Voltage-Dependent Changes In Membrane SHG

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Laser scanning second harmonic generation (SHG) microscopy has shown significant promise for membrane potential imaging with voltage sensitive dyes (VSDs), possessing significant advantages over fluorescence-based imaging modalities. Through simultaneous patch-clamping and non-linear imaging of cells, SHG has been found to exhibit sensitivities to trans-membrane potential that are up to four times better than those obtained under optimal conditions using one-photon fluorescence imaging (Millard *et al.*, 2004). For styryl dyes, while electrochromism is the dominating photophysical mechanism of fluorescence, some ANEP-based dyes display slow SHG voltage responses, suggesting that chromophore membrane reorientation or redistribution may be involved. The mechanism of the SHG response is not entirely understood and necessitates additional study in order to fully optimize this imaging modality. We report on our further investigation of the time dependence of the voltage sensitivity of SHG and simultaneous two-photon fluorescence imaging, using ''fast'' voltage-switching experiments. The response kinetics of resonance enhanced SHG from several styryl dyes developed in our laboratory, including di-3-ANEPPDHQ, as well as di-4-ANEPPTEA and a new fluorinated derivative, have been determined. Voltage-clamped neuroblastoma cells stained with these dyes were imaged with 1064 nm excitation from a mode locked fiber-based

laser source. For SHG, these VSDs were found to exhibit moderately large voltage sensitivities in addition to fast kinetic responses. Our results suggest that voltage sensitive dyes can be developed which have both large SHG signal changes and the requisite speed for use as a practical tool for measuring electrical activity in neuronal systems.
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1504-Pos Board B348
Application of Higher Harmonic Generation Microscopy in Assisted reproductive technologies
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It is known that the quality of the mammal oocytes and embryos greatly influence the outcomes of assisted reproductive technologies. Noninvasive imaging tools with high 3D resolution are thus needed to provide as much information about the embryos as possible. In previous studies, the images of *in vitro* cultured mouse oocytes and embryos were acquired by the harmonic generation microscopy (HGM). Various sub-cellular structures of the whole mouse oocytes and embryos were identified. In our presentation, we will report our study on application of harmonic generation microscopy in assisted reproductive technologies. We use Cr:forsterite laser as the excitation source to obtain the HGM images of mouse oocytes and embryos. First of all, several safety tests are performed to ensure the proper exposure doses of the embryos to the laser. Second, we try to figure out what characteristics in the HGM images of the oocytes and embryos are possibly related to their quality. As to our experimental setup, the HGM signals are collected in both the forward and the backward direction by high N.A. objectives. With limited available working distances, the thickness of the chamber containing the embryos must be within 2 mm. A homemade sterile glass-cover-and-bottom culture dish is designed to meet the need. The dish, containing the embryos, is then placed in a CO_2 stage micro-incubator for HGM observation. Those embryos are later transferred to female mice for pregnancy. The viability of the mouse embryos will be investigated to see if the harmonic generation microscopy can play a role in embryo-selection.

1505-Pos Board B349
Second Harmonic Generation Imaging Microscopy of Ovarian Cancer
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We report the new technique for quantifiable differentiation between normal, high risk, and malignant human ovarian biopsies based on combination of 3D Second Harmonic Generation (SHG) imaging microcopy and Monte Carlo simulation, where we comparatively utilize SHG depth-dependent profiles and bulk optical parameters. We determined that malignant ovaries exhibit larger scattering coefficient, μ_s, and scattering anisotropy, g, than normal tissues. The increased scattering is likely due to higher collagen concentration and fibril density resulting from increased cellular activity. Similarly, the increased anisotropy is consistent with the visual observation of remodeled and more highly ordered fibrils and SHG polarization anisotropy. The underlying structural dissimilarities also lead to significant differences in the measured forward-backward ratio of SHG intensity, which is the metric sensitive to sub-resolution structural effects (local packing of SHG-producing domains). We find that SHG from normal tissues is statistically more forward-directed compared to malignant ovaries, while the high risk ovaries show intermediate behaviors. Data from 100μm below the surface epithelium in the malignant biopsies trend towards the behavior of high risk and normal tissues, suggesting this method is a means of quantifying disease progression into stroma.
Monte Carlo simulations of the photon propagation confirm our experimental data. Here we include the directionality of initially emitted SHG, which is more forward-directed in normal ovaries. Based on our recently developed model using quasi-phase matching conditions relating SHG emission directionality to tissue structure, this finding is consistent with the collagen fibrillar assembly determined through electron microscopy.

1506-Pos Board B350
Analysis Of Multiphoton Imaging Of Thick Biological Scattering Samples
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Non linear optical scanning microscopy has became a useful tool for tissue imaging. Biological tissues are highly scattering media and this lead to an exponentially attenuation of the excitation intensity as the light moves into the sample. While performing imaging of biological scattering tissues in non linear excitation regime, the localization of the maximum 2PE intensity was found to shift closer to the surface [1] and the 2PE imaging depth limit appears strongly limited by near surface fluorescence [2]. In this work we computed the illumination and the photobleaching distribution [3] for different scattering coefficients in order to characterize the effects induced by scattering. An experimental test has been carried out by imaging, with medium numerical aperture objective (N.A.=0.8), thick scattering fluorescent immobile sample (polyelectrolyte gel). Results confirm that in this conditions no photobleaching effects due to scattering occur close to the surface.
[1] J. P. Ying et al, *Appl. Opt.* 38, (1999).
[2] P. Theer *J. Opt. Soc. Am. A.* 23, (2006).
[3] D.Mazza et al, *Appl. Opt.* 46 (2007).

1507-Pos Board B351
SHIM And 2PEM: Getting More Information For Tissue Imaging
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Goal of this communication is to report about a recent study on several different biological samples: bone, cartilage, tendon, tumoral tissues. We show that is possible to couple SHIM (Second-harmonic imaging microscopy) and 2PEM (2Photon excitation microscopy) in a powerful way including polarization properties. SHIM on a laser-scanning system is a unique tool for high-resolution, high-contrast, three-dimensional studies of live cell and tissue architecture. The physical origin of SHG within these tissues is addressed and is attributed to the laser interaction with dipolar protein structures that is enhanced by the intrinsic chirality of the protein helices [2]. Although is a coherent process the multiple scattering through the tissue give us the capability to acquire signal in both backward and forward direction [3]. The orientation of collagen fibers within tissues such as tendons or ligaments is of primary importance. In this study, we propose a simple method based on second harmonic generation (SHG) microscopy to map, pixel by pixel, the orientation of the symmetry axis of the second-order nonlinear susceptibility tensor of collagen fibers of a tendon. The method uses only few images acquired at specific polarizations of the input laser beam. In addition to orientation information, the method would provide polarization independent images and an estimation of the ratio of the nonlinear susceptibility components. This procedure is implemented in both backward and forward scattering pathway. The approach could allow mapping fiber orientation fields, independently of individual fiber contrast in the SHG image. The relationship between images acquired in forward and backward scattering configuration provides more information about sample organization.
[1] P.J. Campagnola et al. Biophys J., 82, 493-508 (2002).
[2] W.R. Zipfel et al. Proc. Natl. Acad. Sci. USA 12, 7075-7080(2003).
[3] A. Diaspro et al. Proc. SPIE 5, 24-31(2002).

1508-Pos Board B352
Vibrational Imaging Based On Stimulated Raman Scattering Microscopy
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We demonstrate a new implementation of coherent vibrational optical microscopy where image contrast is based on stimulated Raman scattering (SRS). SRS detection inherently maps the imaginary part of the third-order nonlinear susceptibility of a molecular vibration. The chemical contrast in SRS microscopy is thus inherently free of nonresonant nonlinear background signal and of spectral interferences between overlapping Raman bands. Experiments are presented that confirm these fundamental advantages of SRS microscopy when compared to coherent anti-Stokes Raman scattering (CARS) microscopy and demonstrate its potential for the noninvasive vibrational imaging of biological systems.

1509-Pos Board B353
Investigating The Network Structure Of Type I Collagen As A Function Of Temperature And Concentration Via Confocal Microscopy
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Three dimensional *in vitro* approximations to extracellular matrix (ECM) are increasingly being used in biophysical experiments investigating cell behavior. One advantage to using collagen I gels as ECM approximations in such experiments is the ability to image the collagen fibers within the gel. This allows simultaneous imaging of cells and their local environment simultaneously with