conference opening

Alexander Savitsky, Wolfgang Becker, DWIH representative

**Anna Savostina,** *German House for Research and Innovation (DWIH) in Moscow,*

**Activities of the DWIH in Moscow. Tools for funding and intensification of German-Russian cooperation**

RUBIN HALL October 3 14.00 – 14.20

Fluorescence and Phosphorescence Lifetime Imaging

Chairmen: Ammasi Periasamy, Elena Zagaynova

RUBIN HALL October 3 14.00 – 14.20

30 min Wolfgang Becker *Becker&Hickl GmbH, Berlin, Germany*

Fluorescence Lifetime Imaging by Multi-Dimensional TCSPC: Advanced Techniques and Applications

30 min Michael Roberts *University of Queensland, Brisbane, Australia*

Using multiphoton tomography with fluorescence lifetime imaging to characterize tissue morphology and exogenous material transport in organs

30 min Klaus Suhling *Kings College, London, UK*

Wide-field Time-Correlated Single Photon Counting FLIM

30 min Elena Zagaynova *Nizhny Novgorod State Medical Academy, Russia*

Tumor metabolism: fluorescence imaging with autofluorophors and genetically encoded sensors

16. 20 – 16. 40 Coffee break

RUBIN HALL October 3 16.40 – 19.50

30 min Dusan Chorvat *Department of Biophotonics, International Laser Centre, Bratislava, Slovakia*

Advanced imaging and spectroscopy of intrinsic fluorophores

20 min **Marina Shirmanova** *Nizhniy Novgorod State Medical Academy*

Measuring viscosity in cancer using molecular rotors and FLIM

15 min Sviatlana Kalinina *University of Ulm, Core Facility Confocal and Multiphoton Microscopy, Ulm, Germany*

Simultaneous NADH-FLIM and oxygen sensing PLIM for metabolic mapping

30 min Thomas Gensch *Research Centre Jülich, Jülich, Germany*

Intracellular ion concentration in living cells and tissue determined by FLIM

30 min Michael Börsch *University of Jena, Jena, Germany*

Observing the rotary motors of F oF1 -ATP synthase at work using single-molecule FRET

25 min Ilya Turchin *Institute of Applied Physics of the RAS, Nizhniy Novgorod, Russia*

Fluorescent small-animal imaging with genetically encoded sensors

20 min Patrick Schaefer *University of Ulm, Ulm, Germany*

Imaging Mitochondrial Function in Alzheimer´s Disease

20 min Anastasia Belova *Institute of Applied Physics of the RAS, Nizhniy Novgorod, Russia*

Fluorescence sensing of hydrogen peroxide level changes under cisplatin treatment of tumor cells

Diffuse Optical Imaging and Clinical Imaging

Chairmen: Valery Tuchin, Heidrun Wabnitz

CONGRESS HALL October 4 8.30 – 11.05

30 min Valery Tuchin *Saratov National Research State University, Russia*

Tissue and cell optical clearing as a tool for enhanced microscopy and imaging: from in vitro to in vivo

30 min Heidrun Wabnitz *PTB Berlin, Germany*

*In-vivo* time-domain diffuse optical imaging of the adult human brain

30 min Karsten Koenig *University of Saarbruecken, Saarbruecken, Germany*

Multiphoton tomography of astronauts

20 min Konovalov Alexander *Russian Federal Nuclear Center – Zababakhin Institute of Applied Physics, Snezhinsk, Russia*

An analytic perturbation model for high-resolution time-domain diffuse optical tomography in the flat layer transmission geometry

20 min Yoko Miura *University of Lübeck, Lübeck, Germany*

Fluorescence lifetime in retinal cell pathology

20 min Martin Hammer  *University of Jena, Jena, Germany*

Pathologic alterations in clinical FLIM at the ocular fundus – lessons learned from two photon FLIM microscopy in vitro

15 min Innesa Ferulova *Institute of Atomic Physics and Spectroscopy, University of Latvia, Riga, Latvia*

Correlation of skin autofluorescence photobleaching rate and the lifetime component

11.05 – 11.30 Coffee break

Super Resolution Microscopy and single molecular detection

Chairmen: Jerker Widengren, Marcel Leutenegger

CONGRESS HALL October 4 11.30 – 13.30

30 min Jerker Widengren *KTH, Royal Institute of Technology, Stockholm, Sweden*

Fluorescence fluctuation and super-resolution techniques - fundamental biomolecular studies and towards clinical diagnostics

30 min Ago Rinken *University of Tartu, Institute of Chemistry, Tartu, Estonia*

Fuorescence-based methods for monitoring lgand binding kinetics to GPCR

20 min Vladislav Shcheslavskiy *Becker & Hickl GmbH, Berlin, Germany*

Scanning near-field optical microscopy enhanced with fluorescence lifetime imaging

20 min Herman Fennema *Nikon Instruments Europe B.V., Netherlands*

Advanced Super Resolution Microscopy Technologies from Nikon

20 min Natalia Klementieva *Nizhniy Novgorod State Medical Academy, Russia*

Fine structure of actin cytoskeleton in cancer cells and tissues unraveled by fluorescence imaging

13.30 – 14.30 Lunch

ADFLIM Plenar

Moderators: Vladislav Shcheslavskiy, Alexander Savitsky

CONGRESS HALL October 4 14.30 – 16.00

45 min Enrico Gratton *University of California, Irvine, USA*

Metabolic changes in cells and tissues revealed by FLIM of intrinsic autofluorescence

45 min Anna Moore *Massachusetts General Hospital, Harvard Medical School, Boston, USA*

Image-guided Precision Nanomedicine for Cancer Therapy

forum opening

CONGRESS HALL October 4 17.00 – 17.40

Chairmen:   
Anatoliy Grigoriev, Yuriy Natochin, Revaz Sepiashvili,   
Vadim Ivanov, Аlexander Gabibov, Alexander Savitsky,   
Arieh Warshel, Christopher Contag,   
Vsevolod Tkachuk, Michail Ostrovskiy, Valeriy Chereshnev,   
Alain Krol, Michael Blackburn

forum Plenar

CONGRESS HALL October 4 17.40 – 19.40

45 min Arieh Warshel

Advancing of computer modeling of biochemical processes at molecular level

45 min Vsevolod Tkachuk

V.P. Demihov –outstanding Russian surgeon, transplantologist, physiologist

30 min Christopher Contag *Stanford University, USA, President of the World Molecular Imaging Society (WMIS)*

Imaging Biology in Living Animals and Humans

welcome cocktail

October 4 20.00 – 21.30

Probe Chemistry

Chairmen: Alexey Bogdanov, Dmitriy Papkovskiy

RUBIN HALL October 5 8.30 – 10.45

30 min Alexander Savitsky *FRC of Biotechnology of the RAS, Moscow, Russia*

SAASOTI as a probe for the super resolution microscopy

30 min Konstantin Lukyanov  *Institute of Bioorganic Chemistry, Moscow Russia*

Towards high-photostability imaging of live cells

30 min Marcel Leutenegger *Max Planck Institute for Biophysical Chemistry, Gottingen, Germany*

Synthetic fluorophores for GSDIM: screening and image analysis

30 min Alexei Bogdanov *Department of Radiology University of Massachusetts Medical School, Boston, USA*

Probes and sensors for near-infrared imaging of enzymatic activity and protein-DNA interactions

15 min Dmitry Gorbachev  *Institute of Bioorganic Chemistry, Moscow, Russia,*

Green fluorescent proteins with long fluorescence lifetime

11. 15 – 11.30 Coffee break

CONGRESS HALL October 5 10.45 – 15.05

forum Plenary session

RUSSIAN MOLECULAR IMAGING SOCIETY FOUNDing CONFERENCE

RUBIN HALL October 5 12.40 – 13.25

13.30-14.30 Lunch

Probe Chemistry(continiation)

RUBIN HALL October 5 14.20 – 15.40

30 min Wolfgang Becker *Becker&Hickl GmbH, Berlin, Germany*

Simultaneous Phosphorescence and Fluorescence Lifetime Imaging by Multi-Dimensional TCSPC and Multi-Pulse Excitation

20 min Dmitriy Papkovskiy *University of Cork, Cork, Ireland*

Imaging of oxygenation and cellular function in 3D tissue models by multiplexed PLIM/FLIM

30 min Ugarova Natalia *Lomonosov Moscow State University*, *Russia*

Firefly luciferase as a probe for imaging and monitoring in living systems

Neuroscience

Chairmen: Alexey Semyanov, Konstantin Lukyanov

RUBIN HALL October 5 16.20 – 18.30

30 min Alexey Semyanov *Institute of Neuroscience University of Nizhniy Novgorod, Russia*

Cellular and subcellular optical imaging in neuroscience

30 min Vsevolod Belousov *Shemyakin-Ovchinnikov Institute of Bioorganic Chemistry, Moscow, Russia*

Thermogenetic stimulation of neurons with single-cell resolution

30 min Ilya Fedotov Lomonosov *Moscow State University, Russia*

Fiber-optic neurointerfaces for fluorescence brain imaging

20 min Olga Ivashkina *Department of Neuroscience, NBICS-center, NRC "Kurchatov Institute", Moscow, Russia*

Neuronal encoding of conditioned signals in the mouse parietal cortex: in vivo two-photon imaging

20 min Franco Klingberg *Thermo Fisher Scientific, Darmstadt, Germany*

New Technologies and Reagents for Live Cell Imaging

Poster session

Moderators: Alexander Savitsky, Wolfgang Becker

RUBIN HALL October 5 8.30 – 13.00

Arseny Aybush *Semenov Institute of Chemical Physics of RAS, Moscow, Russia*

Biological objects visualization in chirped CARS microscopy

Ekaterina Boruleva *Bach Institute of Biochemistry, RSC of the of Biotechnology of the RAS, Moscow, Russia*

The study of endogenous fluorescence of living cells of mammals by FLIM

Varvara Dudenkova *Lobachevsky State University of Nizhny Novgorod, Nizhny Novgorod, Russia*

Quantitative characteristic collagen changes by SHG signal on different biological models

Nadezhda Gurskaya *Shemyakin-Ovchinnikov Institute of Bioorganic Chemistry, Moscow, Russia*

Fluorescence labeling of proteins in live cells using heterodimerization of artificial coiled coils

Natalia Kazachkina *Bach Institute of Biochemistry, RSC of the of Biotechnology of the RAS,, Moscow, Russia , Russia*

Study of fluorescence properties of caspase-3 sensor in tumor cells under the influence of antitumor agents

Sergei Kopanchuk *Institute of Chemistry, University of Tartu, Tartu, Estonia*

Allosteric modulation of peptide ligand binding to Neuropeptide Y receptor Y1 revealed by integrative fluorescence data Global Analyses

Anton Radaev, Elena Koshel *St-Petersburg State University, Russia*

A new lipophilic phosphorescent probe used for two-photon bioimaging

Elena Koshel *St-Petersburg State University, Russia*

New phosphorescent probes based on transition metals complexes for Phosphorescent Lifetime Imaging Microscopy (PLIM)

Alexander Konovalov, Alexander Uglov *Russian Federal Nuclear Center – Zababakhin Institute of Applied Physics, Snezhinsk; Russia*

An analytic perturbation model for high-resolution time-domain diffuse optical tomography in the flat layer transmission geometry

**Rufina Mardanova** *Bach Institute of Biochemistry, RSC of the of Biotechnology of the RAS,, Moscow, Russia*

*Moscow Technological University, Moscow, Russia*

Caspase sensor visualization in 3D cancer cell models

Nadezhda Marynich *Bach Institute of Biochemistry, RSC of the of Biotechnology of the RAS,, Moscow, Russia*

*Lomonosov Moscow State University, Moscow, Russia*

Isolation, purification and characterization of properties of caspase-3 sensors ТR-M5-К и ТR-M6-К

Michael Samtsov *Sevchenko Research Institute of Applied Phisical Problems, Minsk, Belarus*

The fluorescent features of indotricarbocyanine dyes in biotissues

Leonid Shaposhnikov *Bach Institute of Biochemistry, RSC of the of Biotechnology of the RAS,, Moscow, Russia*

*Lomonosov Moscow State University, Moscow, Russia*

The studying of oligomeric state of caspase-3 FRET-sensors TR-M5-K and TR-M6-K

Ilya Solovyev, *Bach Institute of Biochemistry, RSC of the of Biotechnology of the RAS,Moscow, Russia*

*Lomonosov Moscow State University, Moscow, Russia*

New fast photoconvertible protein SAASoti

Alexei Vedyaykin *Research Institute of Nanobiotechnologies, Peter the Great St.Petersburg Polytechnic University,   
Saint-Petersburg, Russia*

Super-resolution fluorescence microscopy for investigation of bacterial cytoskeleton

FLIM-FRET

Chairmen: Enrico Gratton, Claus Seidel

RUBIN HALL October 6 8.30 – 11.20

30 min Yves Mely *University of Strasbourg, Strasbourg, France*

Quantitative and high resolution fluorescence imaging techniques for investigating intracellular interactions and dynamics of HIV-1 proteins

30 min Ammasi Periasamy *University of Virginia, Charlottesville, USA*

Investigation of Prostate Cancer in Live Specimens using FLIM-FRET Microscopy

30 min Claus Seidel *University of Düsseldorf, Düsseldorf, Germany*

Watching structure and dynamics of proteins and protein complexes by high-precision FRET in vitro and in live cells

20 min Piotr Wardega *NanoTemper Technologies RUS LLC, Saint Petersburg, Russia*

Advanced quantitative biomolecular analytics in free solution

15 min Raul Bukowiecki *Max Delbrueck Center for Molecular Medicine, Berlin, Germany; Free University, Berlin, Germany*

Detection of protein misfolding in Huntington’s disease model systems with sensitive TR-FRET-based lifetime imaging

15 min Alexander Goryaschenko *Federal Research Centre “Fundamentals of biotechnology” of RAS, Moscow, Russia*

Rationally designed peptide molecular beacon for highly efficient FRET-sensors

15 min Victoria Zherdeva *Federal Research Centre “Fundamentals of biotechnology” of RAS, Moscow, Russia*

FLIM-FRET of genetically encoded sensor of caspase 3 in tumor xenografts

15 min Darya Smirnova *Lomonosov Moscow State University, Moscow, Russia*

Bioluminescence Resonance Energy Transfer system based on Firefly *L. mingrelica* Luciferase and its application for the Rapid Homogeneous Immunoassay

11.30 –12.00 Coffee break

CONGRESS HALL October 6 10.45 – 16.05

forum Plenary session

13.30-14.30 Lunch

МODERN FLUORESCENT MICROScOPY

Chairmen: Karsten Koenig, Ago Rinken

RUBIN HALL October 616.15 – 17.20

15 min Tatiana Sergeeva *Nizhny Novgorod State Medical Academy, Nizhny Novgorod, Russia*

Analysis of intracellular pH and metabolic changes in cancer cells during apoptosis using FLIM-FRET imaging

20 min Natalia Morozova *Peter the Great St. Petersburg Polytechnic University, St. Petersburg, Russia*

Study of restriction-modification system Esp1396I in *E.coli* at the single-cell level using fluorescence microscopy

15 min Andrei Bogorodskiy *Moscow Institute of Physics and Technology, Dolgoprudny, Russia*

Fluorescent study of *in meso* crystallization of membrane proteins

15 min Natalia Rovnyagina *M.V. Lomonosov Moscow State University, Department of Physics, Moscow, Russia*

Different stages of fibril formation as seen in intrinsic and extrinsic fluorescence

CLOSING OF THE ADFLIM CONFERENCE

RUBIN HALL October 6 17.30 – 18.00

**ABSTRACTS**

**Biological objects visualization in chirped CARS microscopy**

1Aybush A., 1Gostev F, 2Vereshchagin K., 1Titov A., 1Nadtochenko V.

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Recent years chemically selective imaging of complex biological objects is of high interest. The approaches based on multiphoton fluorescence and Raman microscopy are widely exploited. Possessing a whole series of merits CARS microscopy systems are rather complex. In most cases picosecond laser pulses are used for such systems nowadays. Meanwhile, combination of picosecond and femtosecond pulses can bring additional features which stem from spectral characteristics of laser pulses in femtosecond time scale. In this work, we study chirped CARS (c-CARS) two pulse variation of CARS and its potential applicability for biological systems. Pump femtosecond pulse in our c-CARS schemeis stretched up to 10-15 ps while Stokes femtosecond pulse can scan pump frequencies for different delays between pulses. This delay probing allows to reconstruct IR spectrum much faster than in conventional CARS-microscopes thus can be used for 3D scanning systems where time parameter is crucial. Moreover, targeting of IR frequency range from “fingerprints” region to ~4000 1/cm is possible due to fast wavelength tuning of the pump pulse. For several simplesamples we also clarify spectral resolution of the system as well as firm separation of resonance and non-resonance CARS signals.

**Fluorescence Lifetime Imaging by Multi-Dimensional TCSPC: Advanced Techniques and Applications**

Becker W.

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E-mail: [becker@becker-hickl.com](mailto:becker@becker-hickl.com)

Afluorescence lifetime imaging (FLIM) technique for biological imaging has to combine near-ideal photon efficiency, recording of the full fluorescence decay profiles in the pixels, suppression of laterally and longitutinally scattered excitation and emission light, and optical sectioning capability. Moreover, physiological effects occurring during the measurement should not cause artifacts in the recorded decay profiles. The combination of multi-dimensional time-correlated single photon counting (TCSPC) with confocal or two-photon laser scanning meets these requirements almost ideally. FLIM by multi-dimensional TCSPC is based on scanning the sample by a high-repetition rate pulsed laser beam and the detection of single photons of the fluorescence signal returning from the sample. Each photon is characterised by its time in the laser pulse period and the coordinates of the laser spot in the scanning area in the moment its detection. The recording process builds up a photon distribution over these parameters. The result can be interpreted as an array of pixels, each containing a full fluorescence decay curve in a large number of time channels. TCSPC FLIM has got a new push from the introduction of 64-bit data acquisition software. In the 64-bit Windows environment the pixel numbers are no longer limited by the available amount of memory. As a result, images as large as 20148 x 2048 pixels can be recorded. Such images cover the full field of view of even the best microscope lenses at diffraction-limited resolution. The technique can further be extended by recording the photon distribution over additional parameters of the photons. These can be the depth of the focus in the sample, the wavelength of the photons, the time after a stimulation of the sample, or the time within the period of an additional modulation of the laser. Advanced techniques record FLIM Z stacks, multi-wavelength FLIM images, images of physiological effects occurring in the sample, and PLIM (phosphorescence lifetime) images simultaneously with FLIM images.

**Simultaneous Phosphorescence and Fluorescence Lifetime Imaging by Multi-Dimensional TCSPC and Multi-Pulse** **Excitation**

Becker W.

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We present a fluorescence and phosphorescence lifetime imaging (FLIM / PLIM) technique that simultaneously records FLIM and PLIM in confocal or multiphoton laser scanning systems. The technique is based on on-off modulating a high-frequency pulsed laser synchronously with the pixel clock of the scanner, and recording the fluorescence and phosphorescence signals by multi-dimensional TCSPC. FLIM is obtained by building up a photon distribution over the times of the photons in the laser pulse period and the scan coordinates, PLIM by building up the distribution over the times of the photons in the laser modulation period and the scan coordinates. The technique does not require a reduction of the laser pulse repetition rate by a pulse picker, eliminates the need of high pulse energy for phosphorescence excitation, and avoids pile-up problems for the FLIM recording. Due to the excitation of the phosphorescence by multiple laser pulses the sensitivity is orders of magnitude higher than for techniques that use single-pulse excitation at low repetition rate. We demonstrate the technique for the recording of NAD(P)H signals in combination with oxygen partial pressure.

**Fluorescence sensing of hydrogen peroxide level changes under cisplatin treatment of tumor cells**

Belova A.S., Orlova А.G., Balalaeva I.V., Antonova N.O., N.М. Mishina, Zagaynova E.V.

Federal Research Center Institute of Applied Physics of the Russian Academy of Sciences, Nizhny Novgorod, Russia

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In our research were used cell line HeLa Kyoto transfected with the intracellular sensor HyPer2 [1], sensitive to changes in the level of hydrogen peroxide and insensitive to changes of other reactive oxygen species and control cell line, transfected with the intracellular hydrogen peroxide insensitive sensor. Cells were treated with anticancer drug cisplatin. Drug treatment was perfumed in different doses and for different duration. A flow cytometric approach to assess the hydrogen peroxide level under chemotherapy action simultaneously apoptosis marker PE Annexin V and vital dye 7-AAD were used. After drug exposure, the number of viable, early apoptotic, and late apoptotic/necrotic cells was calculated separately. The sensor’s fluorescence response to cytotoxic action was determined for each cells population [2]. Сisplatin at rising concentrations caused a gradual decrease in the number of viable cells and a corresponding increase in the number of early and late apoptotic and necrotic cells in both HeLa Kyoto cell lines as compared to the control. Dose and time dependent increase of hydrogen peroxide level in cancer cells under cisplatin action was demonstrated. Taking into account that HyPer2 reaction was observed for viable, non-apoptotic cells, detected hydrogen peroxide level changes were not the consequence of cell death. The reported study was funded by RFBR according to the research project No. 16-34-01112 мол\_a.

References

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2. A.S. Belova, А.G. Orlova, I.V. Balalaeva, N.O. Antonova, N.М. Mishina, E.V. Zagaynova. Hydrogen peroxide detection in viable and apoptotic tumor cells under action of cisplatin and bleomycin. Photon Laser Med, 1-9 (2016).

**Fluorescent study of in meso crystallization of membrane proteins**

1A. Bogorodskiy, 2,3,4,5V. Polovinkin, 1A. Mishin, 1N. Ilyinsky, 2,3,4,5V. Gordeliy, 6V. Cherezov, 1,7G. Büldt, 8T. Gensch8,

1,2V. Borshchevskiy

1 Moscow Institute of Physics and Technology, Laboratory for advanced studies of membrane proteins, Dolgoprudny, Russia

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8 ICS-4: Cellular Biophysics, Institute of Complex Systems (ICS), Research Centre Jülich GmbH, Jülich, Germany

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With the introduction of membrane protein in meso crystallization 30 years ago by Landau and Rosenbusch, a new era of membrane protein structural research has emerged (1). Since that time this method became associated with a number of major breakthroughs in the field (2) including exceptional successes in structural studies of microbial rhodopsins and G-protein coupled receptors (3). Here we used fluorescence microscopy to study in meso crystallization process of bacteriorhodopsin. Bacteriorhodopsin native fluorescence allows to observe crystallization of unmodified protein while in meso phase provides stable environment for prolonged studies. Several observations using native fluorescence and second-harmonic generation (SHG) of protein crystals provide new insights into the in meso crystallization process. The crystallization starts with formation of microcrystals, followed by growth of a dominating crystal at the expense of smaller ones and formation of a depletion zone around it. These observations demonstrate an Ostwald ripening mechanism of the in meso crystal growth. The depletion zone formed around the growing crystal is consistent with the previously proposed analogy relating in meso crystallization with the crystallization in a microgravity convection-free environment. This work is supported by RSF 14-14-00995.

References

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**The study of endogenous fluorescence of living cells of mammals by FILM.**

1,3 Boruleva E. A., 1 Zherdeva V. V., 1,2 Savitsky A. P.

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Endogenous fluorescence can serve as an indicator of changes in biochemical status of the cells, which to date is demonstrated for a number of endogenous fluorophores. Therefore, the study autofluorescence of cells is an important scientific challenge for fundamental and applied biological research.

In this paper, the FLIM method was used for estimation of the distribution of autofluorescent signal in the range of flavins excitation /emission in HELA tumor cells. The fluorescence microscopy was used for Mitotracker Orange signal visualization. The fluorescent signal was detected using a confocal time resoled fluorescent microscope MicroTime 200 (PicoQuant GmbH, Germany). The images were processed using the PicoHarp and SymPhoTime software (PicoQuant GmbH, Germany). The solid-state lasers with wavelengths 405 nm, 473 nm, 532 nm was used for excitation (PicoQuant GmbH, Germany) and 548/10 nm, 580 nm, 605/15 nm filters (Semrock, USA) was used for registration of fluorescence. The fluorescence spectra of the points was registered using Andor chamber (PicoQuant GmbH, Germany). It was shown that the source of autofluorescent signal was from the organelle morphed structures in the cytoplasm, which are defined as specific staining with mitochondria. There was no contrast bright glow at the point of contacting cells and glow organelle morphed structures on the periphery of the non-contact cells. The results can be applied to the in vivo studies on animals, when the change level of endogenous fluorophores may be an indicator of some pathological processes.

This work was supported by a grant from the RSF 8.07.2015 №15-14-30019

**Detection of protein misfolding in Huntington’s disease model systems with sensitive TR-FRET-based lifetime imaging**

1,2 Bukowiecki R., 1 Dinter F, 1 Schormann E., 3 Shcheslavskiy V.ladislav, 3 Becker W., 1 Wanker E.E.

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Huntington’s disease (HD) is a devastating neurodegenerative disorder caused by an elongated glutamine tract (polyQ) in the huntingtin protein (HTT). The polyQ has to exceed a critical threshold of 35-40 glutamines (Qs) for the pathology to develop. The longer the Q extension the earlier is the disease onset and the more severe is its progression. The N-terminus of HTT is encoded by exon1 (Ex1) that harbours CAG-repeats, which translate into the polyQ. Misfolding and aggregation of HTT constitutes a major hallmark of HD. However, the actual HTT species facilitating aggregation and conferring toxicity is not clearly identified. Sensitive assays to investigate patient material could be promising to develop methods for early therapeutic intervention for HD patients. In this study three different HD models were employed to analyze recombinant human HTT protein in the time-resolved – fluorescence energy transfer (TR-FRET). Those models were: in vitro-aggregated purified protein (Ex1Q23, Ex1Q49), transgenic mice (R6/2), and HEK cells expressing recombinant protein (Ex1Q23, Ex1Q49, Ex1Q79). We identified several antibodies to combine them in FRET pairs and distinguish between monomeric and misfolded protein species. Furthermore, to gain more knowledge about subcellular distribution of aggregates we established a method to visualize TR-FRET in HEK cells based on phosphorescence lifetime imaging (PLIM). In PLIM experiments, we could extrapolate specific lifetime reductions that might correspond to aggregated HTT. With this method we would have a powerful tool to visualize protein conformations more reliably in cells compared to traditional imaging techniques since it is based on two antibodies binding the same antigen. To summarize, with our TR- FRET system it is possible to distinguish between monomeric species from aggregated species in vitro and to identify misfolded HTT with high confidence in different in vitro and in vivo HD models at low nM concentrations. The analyses of biological samples may be helpful to identify aggregation-modifying substances in disease models or clinical studies.

**Advanced imaging and spectroscopy of intrinsic fluorophores**

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Multimodal optical imaging is a promising method for distinguishing of suspected tissues from healthy ones. In particular, the combination of imaging, steady-state spectroscopic methods with time-resolved techniques provides more precise insight into native metabolism when focused on tissue intrinsic fluorescence. On the other side, however, analysis of time-resolved images is a complex task that requires advanced signal processing. This is particularly true when endogenous fluorescence is concerned, due to low intensity and complex determination of individual fluorescence components.

In our contribution we deal with NAD(P)H and flavin fingerprinting in tissues and/or isolated living cells that can be implemented by spectrally-resolved detection, time-resolved detection, or combination of both methods [1]. To obtain spectrally-resolved autofluorescence images related to various states of mitochondrial metabolism and respiration, metabolic modulation was applied in combination with confocal microscopy and spectral detection. Fluorescence lifetime data were recorded using time-correlated single photon counting in single channel and multi-wavelength detection setups using pulsed laser excitation [2]. Comparison of both approaches will be presented, aiming to find an optimized and accurate analytical tool for label-free diagnosis of cells and tissues in their natural environment [3,4].

References

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3. D. Chorvat jr and A. Chorvatova, Laser Physics Letters. 6: 175-193 (2009).

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Acknowledgements

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**Imaging Biology in Living Animals and Humans**

Christopher H. Contag

Stanford University School of Medicine, Stanford, USA

Advances in chemical probes, reporter genes, instrumentation, and integrated imaging strategies are enabling real-time visualization of biological processes in the context of living animals and humans. These advances either build on routine clinical modalities such as MRI, CT, PET and SPECT or incorporate emerging techniques such as in vivo confocal microscopy, photoacoustics, Raman spectroscopic imaging or thermoaccoustics. Preclinical application of these technologies to the study of animal models are leading to improvements in our fundamental understanding of biology, refining our models of human health and disease, and enhancing drug discovery, development and delivery tools. Clinical uses of innovative imaging strategies will lead to earlier detection when diseases are easier to treat, and more precise interventions, ultimately resulting in disease prevention and improved health.

**Quantitative characteristic collagen changes by SHG signal on different biological models**

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The quantitative characteristic of collagen is of primary importance in a wide range of research applications, including the study of different tissue pathology status or their modification during treatment. Studing of collagen is extremely important, because it's the most widely distributed proteins of extracellular matrix in the vertebrate body, constituting about one third of the total body protein. A common way to analyze collagen in tissues is using different special dyes difficult sample processing, that makes it's far from in vivo investigations. Second-harmonic generation (SHG) microscopy came as a powerful alternative modality for imaging fibrillar collagen in different tissues ex-vivo and in-vivo. We studied various samples of normal tissues and pathologic areas. For each change in density stacking, anisotropy degree, swelling or thinning of the fibers, the appearance of a preferred direction or organization and disorganization of collagen fiber bundles, there are the most appropriate approach. The main characterizing methods for quantitative assessment of the state of collagen can be attributed first order statistics (FOS), second order statistics or GLCM (correlation lengths of gray-level co-occurrence matrix), fast Fourier transform (FFT), curvelet transform (CT) and their combination like SHG-to-autofluorescence aging index of dermis (SAAID). Normal coronary arteries and atherosclerotic plaques I, II, III, IV, Va, Vb and Vc types were studied by FFT. We analyzed the normal skin of the rat auricle and under the radiation therapy with both a single dose and fractionated irradiation by SAAID. Assess the condition of the normal bladder to the adenocarcinoma, as well as the change in the state of normal mucosa during radiotherapy with different doses of irradiation was developed by FOS. Moreover normal rectum mucosa was characterized during radiotherapy with different doses of irradiation by FOS. Collagen was quantified by combination of SAAID and FOS on the normal hamster cheek mucosa, under inducing malignancy and further treatment with radiation therapy. Therefore, for characterizing different collagen changes is need to use different approaches or their combination.

**Fiber-optic neurointerfaces for fluorescence brain imaging**

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Integration of advanced fiber imaging methods with optogenetic technologies [1] leads to revolutionary changes in neurosciences, helping confront long-standing challenges in brain research and providing unique tools to study, with an unprecedented spatial resolution and cell specificity, how complex space–time patterns of neural activity relate to higher brain functions. Bundles of optical fibers have been shown to enable imaging of neural structures in living animals [2], multicolor in vivo fluorescence [3] and Raman [4] brain imaging. In a widely used fiber-optic interface for optogenetic work [5], an optical fiber is inserted into a living brain through a guiding cannula right before the experiment. In this work, we demonstrate reconnectable bundles consisting of thousands of optical fibers, which enable, as our experiments show, high-quality image transmission, offering a platform for the creation of implantable fiberscopes for minimally invasive in vivo brain imaging. The concept of reconnectable fiberscopes demonstrated in this work extends the idea of reconnectable implantable single-fiber optical neurointerfaces [6] to the fiber-bundle imaging technology. Experiments on various lines of transgenic mice presented below in this paper verify the performance of this fiberscope as a powerful tool for a chronic, subcellular-resolution in vivo neuroimaging using genetically encoded calcium indicators, neuronal activity markers, as well as axon growth regulators and brain-specific protein drivers in deep regions of awake brain.

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**Correlation of skin autofluorescence photobleaching rate and the lifetime component**

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Non-invasive diagnostic methods in dermatology have several advantages. Optical diagnostics is a fast and relatively easy way for diagnosing skin diseases. In our previous work, results of measurements of skin autofluorescence (AF) photobleaching and influence of photobleaching to fluorescence lifetime of normal skin and pigmented pathology (nevus) were presented [1-3]. Measurements of fluorescence intensity decrease during the laser irradiation and lifetime changes after irradiations are presented in this work. In this study, a 405-nm picosecond laser was used for fluorescence lifetime measurements, and a cw 405-nm laser was used to initiate the photobleaching. Data were collected at 550-nm emission wavelength using a monochromator. The correlation between lifetime changes and photobleaching rates of normal skin and intradermal nevi was estimated. Healthy skin can be characterized by AF intensity decrease of an average of 20 %, while the intradermal nevi showed scattered results ranging from 0 to 30 %. More pronounced lifetime changes during the bleaching process were observed in the case of healthy skin, while the intradermal nevi showed insignificant changes.

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**Intracellular ion concentrations determination in living cells and tissue determined by Fluorescence Lifetime Imaging (FLIM)**

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Ion concentrations are very basic parameters of (bio)chemical reactions and therefore useful to be determined for understanding the functioning of living cells. The estimation of intracellular ion concentrations (absolute and relative) can be non-invasively performed in live-cell fluorescence microscopy using well established, cell-permeable fluorescent indicator dyes as well as genetically encoded sensors. Experiments characterizing both types of sensors for pH, Ca2+ and Cl- as well as measurements applying them for determinations of intracellular pH, Ca2+ and Cl- will be presented. Special emphasis will be given to chloride concentrations in neuronal tissue and the retina.

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Rationally designed peptide molecular beacon for highly efficient FRET-sensors

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The efficiency of the energy transfer in the Forster resonance energy transfer (FRET) based sensor strongly depends on the distance between the donor and acceptor. This distance might be decreased if the linker mainly exists in the beacon conformation. We propose FRET sensor TR-M4-K composed of the fluorescent protein TagRFP and the chromoprotein KFP. The FRET efficiency and dynamic range of measurements in this sensor are increased because of additional structuring by the hydrophobic interactions in the linker, resulting in the beacon-like structure. We analysed the structure of the proposed TR-M4-K sensor for caspase-3 using molecular dynamics, tested several variants of linkers differing in the size of hydrophobic region, and showed that our beacon-like TR-M4-K sensor is hydrolysed by caspase-3 *in vitro* and in living cells after the induction of apoptosis. We found that optimal size of the hydrophopic region is 4 pairs of amino acids. Finally, we proposed a new method for estimating the efficiency of hydrolysis of FRET-sensors by the change of the amplitudes of fluorescence decay components and demonstrated that TR-M4-K sensor has a dynamic range of measurements equal to 4.56 that exceeds the results previously described in the literature.

**Metabolic changes in cells and tissues revealed by FLIM of intrinsic autofluorescence**

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The use of autofluorescence from cells and tissue has a long history going back to the original work of Britton Chance in the ’50 when the spectral properties of the autofluorescence from cells was started to be explored. In mammalian cells, major contributors to autofluorescence arise from NADH and FAD which can be excited at different wavelengths. The interest in this area is because autofluorescence and its excitation and emission characteristics can be related to cellular and tissue metabolism, which is of great interest in the physiology and disease of tissue, specifically in cancer and stem cell differentiation. A relatively recent trend in this area was the introduction by several labs of lifetime methods that are capable of identifying various molecular forms of NADH and FAD. In this presentation we will discuss the phasor approach to FLIM analysis which was shown to be very sensitive to the relative fraction of NADH free and bound to enzymes. However, other autofluorescence molecules can be identifies as well opening up the possibility to better characterize tissue autofluorescence and in particular the results of stress. In this presentation we also discuss a label-free optical imaging technique to assess metabolic status and oxidative stress in human induced pluripotent stem cell-derived cardiomyocytes by two-photon fluorescence lifetime imaging of endogenous fluorophores. Our results show the sensitivity of this method to detect shifts in metabolism and oxidative stress in the cardiomyocytes upon pathological stimuli of hypoxia and cardiotoxic drugs. This non-invasive imaging technique could prove beneficial for drug development and screening, especially for *in vitro* cardiac models created from stem cell-derived cardiomyocytes and to study the pathogenesis of cardiac diseases and therapy.

**Fluorescence labeling of proteins in live cells using heterodimerization of artificial coiled coils**

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Development of transiently binding fluorescent labels for localization super-resolution microscopy is an important task, which could potentially solve the problem of insufficient density of labeling.

Here we proposed novel method of fluorescent labeling of target proteins using heterodimerizing artificial helixes (K/E helixes). The method is based on co-expression of a target protein with K-helix and a fluorescent protein (FP) with E-helix; heterodimerization (coiled coil formation) of K and E helixes provides colocalization of the FP and the protein of interest.

In the literature, several variants of short alpha-helices consisting of 3-5 repeats of specific heptads (for example, KIAALKE for K-helix and EIAALEK for E-helix) are described. Dissociation constant of heterodimeric complexes of these peptides varies in a broad range, depending on number of repeats and amino acid sequences.

First, we constructed a set of plasmids encoding green and red FPs with K- or E-helices of various lengths (3 or 4 repeats) and sequences. K/E-helices interaction was evaluated by colocalization of green and red signals in the pairs, in which first FP had distinct intracellular localization (for example, membrane or chromatin) and second FP had no localization signals. This set of co-transfection experiments allowed to select optimal pairs of K/E-helices, which ensure both good colocalization and fast exchange of the proteins in heterodimers. These pairs were successfully used for live-cell visualization of cytoskeletal proteins in different imaging modalities - confocal microscopy, TIRF microscopy, single molecule localization super-resolution microscopy.

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**Fluorescence lifetimes in the healthy and diseased retina**

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Goal: Fluorescence lifetime imaging ophthalmoscopy (FLIO) may provide a metabolic mapping of the retina by distinguishing different fluorescent compounds such as FAD, Lipofuscin, glycated proteins, and collagen. In order to assess the diagnostic capability of FLIO, we compared mean decay times of patients suffering from age-related macular degeneration (AMD), diabetic retinopathy (DR) macular holes (MH), and Alzheimer’s disease (AD) with that of healthy controls.

Methods: FLIO images were obtained from 25 diabetic patients, 25 AMD patients, 11 patients suffering from AD, 37 MH, and 76 controls. A 30° retinal field was investigated with a Heidelberg Engeneering Spectralis® fluorescence lifetime imaging ophthalmoscope (FLIO), detecting the temporal decay of the fluorescence in a short (498-560 nm; Ch1) and a long (560-720; Ch2) wavelength channel upon excitation with <100 ps (FWHM) laser pulses at 473 nm. The amplitude weighted mean fluorescence lifetime τm was calculated from a three-exponential approximation of the decay. Fluorescence decay times were averaged over a circular, central, macular area and annuli surrounding the macula using a standard grid commonly used in ophthalmology (EDTRS-grid). Optical density of the macular pigment (ODMP) was measured and 3-D morphology data of the retina were obtained by Optical Coherence tomography (OCT).

Results: the controls showed an age-dependence of fluorescence decay. Thus, for comparison with patient cohorts, always an age-matched subset of the controls was used. A study in 48 young healthy subjects (24.4 ± 4.9 years) revealed a significant correlation of the macular τm with ODMP. This finding of the contribution of the macular pigment Xanthophyll to the fast decaying macular autofluorescence is corroborated by the investigation in MH patients. Whereas the lifetime inside the hole, where the retina is lost completely, was similar to that of the peripheral retina (ch1: 238 ± 64ps vs. 284 ± 77ps, ch.2: 275 ±49ps vs. 274 ±41ps), the decays in the retina surrounding the hole, which contain Xanthophyll, were significantly shorter and resembled that of intact maculae from control subjects: (ch1: 181 ± 78ps vs. 159 ± 43ps, ch.2: 223 ±48ps vs. 203 ±33ps). Predominantly in ch. 1, patients with diabetic retinopathy sowed longer decay times than controls (327±148ps vs 203±75ps, inner ring of the ETDRS grid, p<0.05). In a logistic regression model, a combination of independent FLIO parameters discriminated the patients from the controls with a sensitivity of 76.5% and a specificity of 78.6%. In AMD patients, an extension of the fluorescence decay times was found rather in ch. 2. In patients, suffering from Alzheimer’s disease, we found a correlation of the relative abundance of the intermediate decay component (q2) in the long-wavelength channel with results of neuropsychological testing (MMSE, p=0.008) as well as the pathognomonic concentration of -protein in the cerebro-spinal fluid (p=0.039).

Conclusions: FLIO may discriminate AMD and diabetic patients from controls. Comparison with ex vivo measurements indicates lipofuscin, melanin, FAD, collagen, and, eventually, NADH as major fluorophores opening a possibility for metabolic fundus imaging by FLIM. The shortest lifetime τ1 may be addressed to the macular pigment, τ 2 to the RPE and the retina, and 3 to connective tissue and the lens.

**Neuronal encoding of conditioned signals in the mouse parietal cortex: in vivo two-photon imaging**

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A common way to study memory of neuronal representations in the rodent neocortex is Pavlovian conditioning to a discrete sensory stimulus. However, in natural environment associative memories are complex and involve integrated conditioned stimuli (CS) consisting of different sensory modalities. Very little is known about how neuronal populations in neocortex code complex stimuli during long-term memory formation and retrieval. Previously we have developed a behavioral paradigm for multisensory compound CS fear conditioning in mice that mimics complexity of natural learning. In the present study, we examined encoding of different components of the compound CS by neurons of the mouse parietal cortex. To map neuronal responses we used transgenic mice with the expression of the EGFP controlled by the c-fos promoter. We trained mice in a fear-conditioning task to a compound cue that consisted of auditory (tone) and visual (blinking light) components. Control mice were presented with the compound CS, without the footshock. A week later, we performed three sequential retrieval sessions during which mice received separate auditory and visual components of the compound CS and the compound CS itself. To monitor retrieval-induced neuronal activation we performed two-photon in vivo imaging of fos-EGFP expression in the parietal associative cortex 90 minutes after each test. Based on the intensity of EGFP fluorescence, all registered neurons were divided into the two groups - high- and low-expressing. The number of high-expressing neurons increased in the trained mice compared to the control mice in all retrieval sessions, while the number of low-expressing neurons decreased in all the sessions. Next, we analyzed neurons that showed high fos-EGFP expression only in one of the retrieval session. We found three activation specificities of such neurons: light-related, sound-related and compound CS-related neurons. The number of light-dependent high-expressing neurons increased in the trained mice compared to the control mice. The number of sound-dependent and compound CS-dependent neurons was equal in the trained and control mice. Taken together our data suggests that coding of complex associative memory in the parietal cortex involves at least three neuronal assemblies with different response specificity to the components of the compound CS.

**Study of fluorescence properties of caspase-3 sensor in tumor cells under the influence of antitumor agents.**

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Caspase-3 plays an important role in regulation of life activities of mammal cells.

The present work is devoted to study of fluorescence properties of TR23Ksensor for caspase 3 *in vitro* and *in vivo* under the influence of antitumor treatment.

Hep-2 cells expressing TR23K (Hep-2/TR23K) or TagRFP (Hep-2/TagRFP) were obtained by means of transfection usingpLVTR23K or pLVTagFRP, correspondently, and were used in this work. The *cis*-dichlorodiammineplatinum (II) (cDDP) and etoposide were used as cytotoxic agents.

*In vitro* IC50 for cDDP was 4.5 g/ml and 1.3 g/ml after 24h and 48h of cells incubation, correspondently. IC50 of etoposide was 8.9 g/ml and 2.6 g/ml, correspondently. The fluorescence life time of treated Hep-2/TR23K cells increased from 1.8 to 2.5 nsand the cleavage of the sensor has been confirmed by Western blotting.

For *in vivo* studies fluorescent tumours were obtained by injected *s.c.* into nu/nu mice of 5 million of Hep-2/TR23K or Hep-2/TagRFP cells. cDDP was used alone (7.5 mg/kg i.v.) or in combination with etoposide (DDP - 5 mg/kg i.v. immediately thereafter etoposide was administered in the dose of 5 mg/kg i.p.). Chemotherapy (CT) was done once on the 44th day of the tumour growth.

Fluorescence analysis of tumours was performed *in vivo*1 day before and 1, 3-4 and 6 days after CT. After the last measurement of fluorescence the tumours were subjected to histological examination.

The increase of the fluorescence intensity of the Hep-2/TR23K tumours was observed 24h after CT. The fluorescence life time increased from 1.67±0.07 to 2.02±0.04 ns.

The corresponding changes of fluorescence life time of Hep-2/TR23Kcells under cytotoxic treatments were caused by activation of effectors’caspases.

This study was supported by Russian Science Foundation (project 14-08-01017 А).

**Fine structure of actin cytoskeleton in cancer cells and tissues unraveled by fluorescence imaging**

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Actin, one of the most abundant and highly conserved protein, is involved in many cellular activities, either in normal physiology or in diseases. Сell adhesion, migration and invasion driven by actin dynamics play an important role in tumorigenesis. In this light, the reorganization of actin cytoskeleton may be considered as a marker of metastatic potential and drug sensitivity of cancer cells. However, fine structure and remodeling of microfilaments within tumor tissue still remains unclear. The emerging super-resolution microscopy fluorescence techniques overcome the diffraction limit and enable to fully understand actin network. Here, we proposed a simple way to visualize endogenous actin within cancer cells and tissues at ultra-high resolution by fluorescence imaging based on recently introduced SiR-actin dye. First, we established imaging conditions favourable for SiR-actin blinking. Then we studied actin dynamics in cancer cells in response to chemotherapeutic agents by SiR-actin based single-molecule localization microscopy. A marked increase in size of focal contact sites, their convergence as well as stress fibers shortening were observed in cells after incubation with taxol. In case of cytochalasin D treatment, short actin fibrils along with point-like structures less than diffraction barrier were found. Next, we established the mouse models of colon and lung carcinomas to move to tissue imaging. We have found out actin cytoskeleton in tumors significantly differs from that in vitro. There was an omnidirectional actin mesh composed of curved stress fiber-like structures in tumor tissues. Interestingly, we detected no resemblance between tumors and normal tissues of close origin.

To summarize, we developed protocol of actin visualization using SiR-actin staining suitable for single-molecule localization microscopy. This allowed us to observe how dynamic actin cytoskeleton underwent a change upon chemotherapy. We for the first time revealed a complex network of thick curved bundles of actin in tumor tissues by super-resolution fluorescence microscopy. Potentially, the proposed method can be adapted for imaging of fine actin structure in clinical samples.

This work was financially supported by the Russian Science Foundation (project # 14-25-00129).

**New Technologies and Reagents for Live Cell Imaging**

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„A picture is worth a thousand words“. This idiom refers to the notion that a complex idea can be conveyed with just a still image. Now imagine what a story a video can tell?! A fixed sample reveals many details about a biological specimen but does not give insight into the dynamics of interactions. Adding another dimension such as time provides a great opportunity to study extra- and intracellular events. However, many challenges exist when preparing a time-laps experiment on high-end instruments. Therefore, we have development our second generation of automated imaging systems that enable an easy setup of live-cell experiments. One of the most important factors in live-cell imaging is the environmental control. There is little room for error or variation when it comes to keeping conditions such as gas content and temperature constant. Hence, atmospheric conditions should be controlled and monitored constantly during time-laps. Fluorescent imaging represents another level of complexity as fluorophore labeling or endogenous expression of fluorescent markers needs to be optimized. We have developed new Molecular Probes® reagents that enhance fluorophore stability, increase imaging quality, monitor cell health and indicate various changes within the cell. In summary, live-cell imaging is now available for everyone and can easily become a routine method in your lab. It definitely should be considered when studying dynamic cellular mechanisms.

**An analytic perturbation model for high-resolution time-domain diffuse optical tomography in the flat layer transmission geometry**

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The paper develops a perturbation model for time-domain diffuse optical tomography in the flat layer transmission geometry. The uniqueness of this model is that as measurement data it uses the time-resolved optical projections (Konovalov A.B. et al. Proc. SPIE 80880T, 2011). These projections are defined for the individual counts of the temporal point spread function as relative disturbances in the photon fluxes, which are caused by optical inhomogeneities. Minimizing the time-gating delay of receivers, we can make the banana-shaped zones of the photon trajectory distributions ultimately narrow and thus minimize the spatial resolution. We derive an analytical representation of the weight functions which model the imaging operator by using a diffusion approximation to the radiative transfer equation and the perturbation theory by Born. To evaluate the weight functions for the flat layer transmission geometry, we first derive analytical expressions for the case of a semi-infinite scattering medium by using Green’s function of the nonstationary diffusion equation and then apply the original method of an equivalent inverse source (Konovalov A.B. and Vlasov V.V. Quantum Electron. 44, 719, 2014). The efficiency of our model is demonstrated through a numerical experiment wherein the rectangular scattering phantoms with absorbing and scattering inhomogeneities are recovered using the modified multiplicative algebraic reconstruction technique (Konovalov A.B. et al. Quantum Electron. 38, 588, 2008). The inhomogeneities form periodic spatial structures in order to test the spatial resolution of the method. We investigate the dependence of the resolution limit on the phantom thickness. It is shown that the transverse resolution is approximately equal to 2.5 mm for a 8 cm-thick phantom and close to 1.0 mm when the phantom thickness is 2 cm. With respect to the longitudinal resolution, the same values are 3.5 and 1.5 mm respectively. In our opinion, the reconstruction model similar to the proposed one can be constructed for time-domain fluorescence molecular tomography too. In this case an analogue of the time-resolved optical projection can be the ratio of fluorescence flux to exciting radiation flux measured for individual counts of diffuse fluorescence temporal response.

**Allosteric modulation of peptide ligand binding to Neuropeptide Y receptor Y1 revealed by integrative fluorescence data Global Analyses.**

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Implementation of fluorescence methods in studies of ligand binding to their receptors opens new possibilities to characterise these processes. One of the potential approaches is the detection of changes of the fluorescence anisotropy (FA) and/or total fluorescence intensity (TFI) signals upon binding reaction. However, to achieve significant changes in the FA/TFI signal, some requirements need to be met – the concentration of receptor binding sites as well as the dissociation constant of the interaction should be in the same order as the fluorescent ligand’s concentration. We have used FA assay to investigate ligand binding properties to Melanocortin 4 (MC4) receptor (1). Implementation of budded baculoviruses (BBV) that display G protein-coupled receptors on their surfaces significantly increased sensitivity and temporal stability of this assay (2). For the first time we demonstrate the applicability of BBV experimental setup to study Y1 receptor system. Here we used TAMRA-PYY, an Y1 receptor specific fluorescent peptide ligand, as a reporter ligand. Besides real-time monitoring of FA signal changes, up to 5 fold decrease in TFI signal was observed within TAMRA-PYY binding to the Y1 receptor. Pharmacological characterization of Y1 receptors with receptor-specific unlabelled ligands gave the rank order of potencies consistent with previously reported values. Additionally, allosteric heterogeneous interactions were revealed as koff values of TAMRA-PYY differed more than 7 times depending on the nature of dissociation initiated ligand. These observations provide evidence for similar allosteric receptor-ligand binding mechanism as previously shown for MC4 receptors (3). With the presented fluorescent ligand, it was possible to follow interaction process by the change of tracer fluorescence lifetime and we suppose that implementation of FCS with BBVs that display receptors on their surfaces opens additional possibilities for assessment of receptor-ligand interaction mechanisms.

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**A new lipophilic phosphorescent probe used for two-photon bioimaging**

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Metal-based phosphorescent probes are useful tools for bioimaging, including two-photon microscopy. In this study, we present investigation of a new phosphorescent homoleptic alkynyl gold(I) cluster, (AuC2R)10 (R – 2,6-dimethyl-4-heptanol) (Koshevoy et al., 2012), which demonstrates a selective staining of lipid droplets in animals tissues and human cells.

We revealed rather high quantum yield (0.66) and a typical for gold-clusters phosphorescence lifetime of the probe in methylene chloride (0.91 µs). We show that one- and two-photon emission spectra at 405 nm and 710 nm, respectively, do not depend on the excitation type and have a maximum fluorescence at 570 nm.

For microscopic experiments *ex vivo*  we used different models: i) cryosections of fixed tissues of different animals (mouse, chicken, and pigeon) enriched by either nonpolar (skin, visceral adipose tissue, liver ) or polar (brain, liver) lipids; ii) fixed Hep G2 cells fed by neutral lipid emulsion (Lipoidol Ultra Fluid) to form lipid droplets. The samples were treated by the solutions of the probe in isopropanol:PBS (1:1 by volume) mixtures (1 mg/ml). The confocal microscopy analysis of all samples demonstrated selective staining of lipid droplets by the probe. To prove the specificity of the probe towards lipid droplets, we also analyzed its colocalization with Oil Red O and Nile Red, which showed high Pearson’s correlation (>0.77) and overlap (>0.84) coefficients for all samples. For time-resolved phosphorescence imaging of stained tissues, Leica TCS SP8 MP (Leica Microsystems, Germany) equipped with fs Ti:sapphire laser (Chameleon Ultra I, Coherent Inc., USA) and Simple-Tau 150 TCSPC system (Becker&Hickl GmbH, Berlin, Germany) was used. Phosphorescence lifetime imaging measurements yielded the probe phosphorescence lifetime around 0.8-1.2µs.

We believe that the combination of high quantum yield and lipid affinity of this phosphorescent complex can make it attractive for one- and two-photon bioimaging applications. The implementation of new phosphorescent dyes would provide deeper insight into adipocyte metabolism as well as development of diseases associated with lipid metabolic disorders.

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**New phosphorescent probes based on transition metals complexes for Phosphorescent Lifetime Imaging Microscopy (PLIM)**

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Simultaneous fluorescence and phosphorescence lifetime imaging microscopy (FLIM and PLIM) of living cells makes it possible to obtain a more detailed picture of intracellular homeostasis. In particular, *in situ* measurement of oxygen concentration in biological objects and its effect on the metabolism of the cells can be measured with PLIM based on organometallic phosphors. This work presents the results obtained using novel phosphorescent probes such as Pt or polynuclear Au-Cu complexes conjugated with human serum albumin (HSA) for delivery into cells. The two Pt-probes: Pt1 ([Pt(C11NH8)(PPh3)Cl]) and Pt2 ([Pt(C11NH8)(C3NH2(C2H4SO3Na)2)(C2PhCOOSu)]), conjugated with monomeric HSA and aggregated noncovalent adducts of Au-Cu alkynyl-diphosphine complex Au1, [{Au3Cu2(C2C6H5)6}Au3(PPh2C6H4PPh2)3] with HSA, were prepared and their internalization into live cells was studied.

Cell cultures (HeLa and CHO, 15x104 cells/ml) were grown in the glass bottom dishes and slides, and later incubated with the probes in concentration of 0.3mg/ml for 24 hours. Electroporated cells were used as a positive control of internalization of the probes into cells. For simultaneous FLIM of NAD(P)H and PLIM of the probes we used a Nikon TE 2000 microscope equipped with the confocal scanner DCS-120, Simple-Tau 150 TCSPC system and a 405nm pulsed diode laser (Becker&Hickl GmbH, Germany). The variation of oxygen concentration in the cell containing solutions was performed by adding Na2SO3.

Both of Pt-HSA probes successfully interact with untreated HeLa and CHO cells and display 6 µs lifetime in normoxia (20-21% oxygen). The Au1-HSA label does not infiltrate into untreated HeLa and CHO cells but penetrate into electroporated ones as the Pt-HSA probes do. Under the oxygen reduced conditions the Pt-probes lifetime increased to 8 µs that indicates the prospective to use these conjugates for oxygen monitoring in live cells via PLIM method.

The work is supported by Saint-Petersburg State University research grants #1.37.153.2014 and #1.50.1043.2014. The equipment of the “Chromas” SPbU Recourse Centers (Saint-Petersburg, Russia) and The Max Delbrück Center for Molecular Medicine (Berlin, Germany) were used.

**Synthetic fluorophores for GSDIM:screening and image analysis**

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Single-molecule localization microscopy such as GSDIM [1] and STORM [2] overcomes the diffraction limit by analyzing an image sequence of blinking fluorophores for extracting the positions of individual fluorophores. Based on these positions a high-resolution image of the fluorophore distribution in the sample can be reconstructed. The spatial resolution of the reconstructed image is not limited by diffraction but by the precision of the position estimates and the density of localized fluorophores. The quality of the reconstructed image thus relies chiefly on photo-physical and photo-chemical properties of the fluorophore, which have been studied for a number of fluorophores earlier, see for instance [3,4].

In this study we investigated major photo-physical properties of a large number of synthetic fluorophores under typical imaging conditions. For this purpose, we immunostained the nuclear pore protein NUP153 in Vero cells by secondary antibody labeling, imaged these fixed cells with a commercial GSDIM microscope by Leica and analyzed the reconstructed GSDIM images and the event lists of fluorophore localizations. We screened the synthetic fluorophores in different buffer solutions: an imaging buffer containing glucose oxidase (Glox) enzyme and -mercaptoethylamine (MEA); phosphate-buffered saline (PBS); for some fluorophores Mowiol as well. GloxMEA is a typical oxygen-depleting thiol-containing imaging buffer that improves the performance of many fluorophores decisively for localization microscopy, see for instance [5,6]. We were also interested in fluorophores achieving good performance in PBS because these fluorophores could be potentially applied for live cell imaging.

We analyzed the localization events to estimate key parameters such as the lifetime of the bright state (average duration of blink events), the average number of detected photons per blink event, the photo-bleaching rate and the number of localizations per nuclear pore complex. We sorted the reconstructed images for increasing contrast and sharpness. We then combined the criteria into an overall performance index for each fluorophore and imaging buffer.

The presented method allowed us to investigate a large number of fluorophores in immunostained fixed cells and compare their suitability for GSDIM microscopy. Not surprisingly, we found numerous fluorophores applicable with the GloxMEA buffer. However, we also found several fluorophores that perform well in PBS. For both GloxMEA and PBS buffer, we found spectrally different fluorophores, particularly useful for multi-color imaging.

Acknowledgements

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**Towards high-photostability imaging of live cells**

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A significant problem of fluorescence microscopy is photobleaching of the probes. Many advanced imaging modalities such as time-lapse and 3D microscopy, single molecule detection, super-resolution microscopy and fluorescence correlation spectroscopy strongly suffer from insufficient photostability of fluorescent proteins. Unfortunately, our knowledge on molecular mechanisms of photobleaching in fluorescent proteins remains fragmentary. At the same time, high-throughput screening for photostable variants is technically challenging.

In this talk, I will discuss our efforts to enhance photostability of fluorescence signal in live cells. An important step forward in understanding and overcoming of the GFP photobleaching was discovery of oxidative green-to-red photoconversion (“redding”) based on electron transfer from excited GFPs to intracellular electron acceptors. It was realized that oxidative redding can represent a major pathway of GFP photobleaching in live cells. We found that suppression of redding by optimization of imaging media composition is a simple and efficient way to increase GFP photostability. Also, we performed mutagenesis of key residues potentially participating in electron transfer from the chromophore to an external acceptor. As a result, variants with improved photostability were generated.

A potential ultimate solution of the photostability problem is fast replacement of the bleached fluorophore molecule by the fresh one. To this end, we developed a new method of protein labeling based on specific binding of fluorogens with bacterial lipocalin mutants. Due to reversible binding of dyes in the protein pocket, very high photostability of fluorescence signal can be achieved. Labeling with low concentrations of fluorogens provides a simple way to single-molecule detection-based super-resolution fluorescence microscopy.

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**Caspase sensor visualization in 3D cancer cell models**

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We have used cancer cell line of adenocarcinoma of the human larynx HeP-2 to visualize the caspase sensor. Trasfection with genetic construct has been carried out using lentiviral particle (Evrogen, Russia). A new technique of spheroid growing of HeP-2 TagRFP and HeP-2 TagRFP/KFP has been developed. In 5 days the spheroids were centrifuged and then were shot fluorescence of the layers in increments of 2 microns. A time-resolved confocal microscope Microtime 200 (PicoQuant, Germany) was used to visualize caspase activity by FLIM-FRET technique. Image reconstruction was performed using ImageJ software.

This work was supported by Russian Science Foundation, grant № 15-14-30019.

**Quantitative and high resolution fluorescence imaging techniques for investigating intracellular interactions and dynamics of HIV-1 proteins**

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Quantitative imaging techniques, such as FLIM, FCS and FCCS, and RICS are powerful tools to monitor the dynamics and interactions of proteins in live cells. We used these tools to monitor the intracellular fate of the nucleocapsid proteins (NCp7) of the Human immunodeficiency virus type 1 (HIV-1) that are likely released in the late steps of reverse transcription. We found that NCp7 localizes mainly in the cytoplasm and the nucleoli, where it binds and diffuses with ribosomal RNAs (1). The binding of NCp7 to ribosomes was further evidenced by the intracellular co-diffusion of NCp7 with a protein of the large ribosomal subunit. Moreover, FLIM images of micro-injected NCp7 labeled by an environment sensitive probe confirmed the nature of its cellular partners (2). Finally, super-resolution PALM allowed us accurately defining the distribution of NCp7 in the nucleoli domains and track fluorescently labeled HIV-1 pseudo particles.

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**Isolation, purification and characterization of properties of caspase-3 sensors ТR-M5-К и ТR-M6-К**

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Biosensors on the caspase-3 (effector protease which is responsible for the hydrolysis of cellular proteins during apoptosis) allow to evaluate the efficiency of antitumor drugs designed to activate caspase-dependent apoptosis.

In our laboratory sensor TR-M4-K was designed, consisting of TagRFP and KFP proteins, coupled with amino acid linker containing a caspase- 3 recognition site DEVD. The linker has a rigid structure formed by hydrophobic amino acids. It was shown that the sensor is effectively hydrolyzed by caspase-3 *in vitro* and in living tumor cells. To optimize the linker length, sensors TR-M5-K and TR-M6-K, with addition of one or two hydrophobic amino acid pairs to the linker respectively, were obtained.

Purification of these sensors was carried out by fractional precipitation followed by ion exchange chromatography. Degree of maturation and purity was determined by SDS-PAGE. The oligomeric state of the sensors was determined by dynamic light scattering and gel filtration. It has been shown that both TR-M5-K and TR-M6-K are in solution in the form of macromolecular assemblies.

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**Image-guided Precision Nanomedicine for Cancer Therapy**

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Precision medicine targets the molecular basis of disease and as such is improving cancer treatment. The use of a patients’ specific biological information to guide their individual treatment will improve outcomes by addressing the precise nature of their disease. At the same time, the ability to image drug delivery in real time in patients can predict their response to therapy and significantly improve survival.

The potential of small non-coding RNAs in precision medicine is indisputable, considering that this mechanism can be used to silence virtually any gene, with single-nucleotide specificity. Small interfering RNAs (siRNA) and microRNAs have emerged as regulators of post-transcriptional modification of gene expression and are poised to dramatically impact cancer therapy. Molecular imaging can provide vital information about the delivery of RNA-based drugs to the tumor site and assist in evaluating the therapeutic efficacy. This presentation will focus on pre-clinical development of nucleic acid-based cancer therapies, imaging their delivery and application to the treatment of metastatic disease.

**Study of restriction-modification system Esp1396I in *E.coli* at the single-cell level using fluorescence microscopy**

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Restriction-modification (RM) systems are widely used by bacteria for protection against foreign DNA due to the action of site-specific restriction endonuclease (RE). On average, the presence of an RM system reduces the efficiency of bacteriophage infection by three orders of magnitude. RM systems genes are subjects to horizontal transfer and are often carried on plasmids.

To study RM system at the single-cell level,a fluorescently labeledC-protein-dependent RM system Esp1396I encoding RE: mCherry and MT: Venus fusion proteins was constructed. Sucha system is functional and both protein fusions are stable in *E.coli* cells.

Fluorescent labeling of RM enzymes allowed us to measure concentrations and to observe variability of RE and MT fusion proteins in single bacterial cells.

We were able to follow the dynamics of RM enzymes appearance and accumulation after transformation of naïve *E.coli* cells with a plasmid carrying the engineered RM system.A significant delay between MT and RE accumulation and difference in dynamics of their accumulation were directly observed for the first time.The methodology developed in current workallows to study the dynamics of protein levels in a singlebacterial cells and can also be used to study the correlation between levels of RE and MT in individual cells and how these levels affect the probability of successful phage infection.

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**Imaging of oxygenation and cellular function in 3D tissue models by multiplexed PLIM/FLIM**

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Molecular oxygen (O2) plays a multitude of important roles in cell and tissue function. We have developed a family of cell-penetrating phosphorescent probes(small molecule and nanoparticle structures) and applied them to study several different 3D tissue models by multiplexed high-resolution Phosphorescence Lifetime Imaging Microscopy (PLIM). In particular, we showed that deoxygenation of aggregates/spheroids of PC12 (rat pheochromocytoma) and HCT116 (human colon cancer) cells is significant and depends on the method of their preparation, howevercells remain viable and respond to treatment with metabolic effectors. For the embryonic rat brainneurospheres and cultured tissue slices, O2 PLIM was multiplexed with fluorescent imaging of cell type, viability and proliferation markers, and with immunofluorescent staining. Ex-vivo analysis of mouse bladder epithelium revealed marked intracellular gradients of O2in giant umbrella cells (up to 40-50 mM across the cell or 0.6 mM/mm), which may play important roles in their functioning. We also observed a decreased respiration of colonocytes in the colitic colon tissue, compared to normal, and correlated tissue O2 with ROS generation. The results demonstrate high utility of cell-penetrating O2 probes and PLIM method for life science research.

**Investigation of Prostate Cancer in Live Specimens using FLIM-FRET Microscopy**

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The treatment and diagnosis of prostate cancer is a much debated subject, revolving about questions about early diagnosis in the context of PSA (Prostate-specific antigen) tests, when to treat, the heterogeneity of biopsy samples and when surgery is warranted. One of the underlying problems is the lack of a sensitive test, which will reduce some uncertainties and aid clinicians to diagnose and decide with which therapeutic option to pursue. Label-free optical imaging of endogenous fluorophores is a powerful non-invasive method, providing direct in-vivo information of the cellular microenvironment and changes in metabolic activity under physiological and pathological conditions by following tissue or cellular metabolic signatures through these fluorophores. This presentation focuses on to monitor the endogenous molecules NADH, FAD and Trp to understand the metabolic activity in prostate cancer living cells using Förster resonance energy transfer (FRET) and fluorescence lifetime imaging microscopy (FLIM) techniques. Glucose uptake and glycolysis proceeds about ten times faster in cancer than in non-cancerous cells or tissues. Therefore, we assessed the glycolytic activity in the prostate cancer in comparison to normal cells upon glucose stimulation by analyzing the NADH and Trp lifetime distribution and efficiency of energy transfer (E%). Furthermore, we treated the prostate cancer cells with 1╣M Doxorubicin, commonly used chemotherapeutic for cancer treatment due to its potency, rapid uptake and ability to attack rapidly dividing cells, regardless of the cell cycle phase. Increase in NADH a2% as an indicator of increased glycolysis in Prostate cancer cells and increased E% between Trp and NADH was seen upon glucose stimulation for 30min. The magnitude of the NADH a2% and E% distribution was higher in prostate cancer cells as compared to the normal cells. Upon treatment with Doxorubicin decrease in cellular metabolism at 15 and 30 minutes was seen. Hence, optical redox ratio and Trp lifetimes can be used as a biomarker to understand metabolic activity in prostate cancer and upon chemotherapeutic interventions.

**Fluorescence-based methods for monitoring lgand binding kinetics to GPCR**

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G protein coupled receptors (GPCRs) constitute a family of receptors that transduce signals into cells via guanosine nucleotide binding regulatory proteins (G proteins). As GPCRs mediate different stimuli and modulate various signal transduction pathways, they are also important targets of drug development for treatment of various diseases. As ligand binding dynamics is an essential factor for its action, the development of novel drugs has to pay attention also to their kinetic properties. Several novel fluorescence-based methods that allow on-line monitoring of ligand binding kinetics have been developed and implemented for drug discovery studies during the last decade. We have implemented the fluorescence anisotropy (FA) and fluorescence intensity (FI) based assays to investigate fluorescent ligand binding to different GPCRs [1]. The utilization of budded baculoviruses that express G protein-coupled receptors on their surfaces have significantly increased sensitivity and applicability of these assays [2]. The assay system has already implemented for different GPCRs, like melanocortin (MC4R), serotonin (5-HT1AR), dopamine (D1DAR) and neuropeptide Y (NPY1R). The real-time monitoring of ligand binding to the receptors helps to understand mechanism of reaching of equilibrium for each particular ligand and possible mechanisms of its regulation. The work has been financed by the Estonian Ministry of Education and Science (IUT20-17) and by the European Regional Development Fund (EU48695)

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**Using multiphoton tomography with fluorescence lifetime imaging to characterize tissue morphology and exogenous material transport in organs**

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Traditionally, the extent of organ disease and the transport of compounds in the body have been determined by a combination of histology, clinical chemistry and pharmacokinetic methods amongst others. A revolutionary change is being able to image events and processes in organs in vivo and ex vivo using multiphoton technologies combined with fluorescence lifetime imaging microscopy. Further advances are now occurring with the combining these technologies with confocal reflectance, confocal photoacoustic, second harmonic and third harmonic imaging. In this work, we describe some examples of altered renal and liver pathology as a result of various diseases processes and chemical injury together with the mapping of the kinetics of exogenous and endogenous solutes, cells and nanosystems administered into the body or applied to the skin *in vivo* and *ex vivo*.

**The fluorescence recording features of indotricarbocyanine dyes in biotissues**

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Further development of personalized therapy for the oncologic patients is associated with the creation of multifunctional substances selectively accumulated within neoplasms, featuring the cytotoxic properties, and enabling localization of malignant tumors. Optical diagnostic procedures may be realized using the fluorescent compounds characterized by considerably different properties in tumors and in normal tissues. Most promising for such procedures are the polymethine dyes (PD) having their absorption and fluorescence bands in the spectral region, where biological tissues are transparent. In this region the lowest light absorption level of the tissues is attained at the deepest penetration of radiation.

It is essential to elucidate the difference in the spectral-luminescent properties of the luminophors in tumors and in normal tissues. This work presents a study of a new indotricarbocyanine dye examined with this purpose.

The possibilities of visualizing experimental tumors have been tested on Nu/Nu line mice with the use of a FMT 4000 diffuse fluorescence tomograph (Perkin Elmer, USA) at the excitation wavelength 680 nm and the recording wavelengths 770-800 nm. The results obtained during FMT studies have been compared to those derived by spectrometry of isolated organs and tissues using a fiber-optic spectrometer. It has been found that the intrinsic fluorescence effect of biotissues in the process of fluorescence recording over the spectral range 710-900 nm in vivo may be lowered when using for excitation laser sources with the wavelengths above 676 nm. It is demonstrated that, to ensure proportionality of the photosensitizer fluorescence signal to its concentration in vivo, the light penetration depth and the form of fluorescence spectra should be controlled with the introduction of several photosensitizer concentrations. As demonstrated by the results, the photodynamic therapy method is rather effective for recording of the accumulation and removal pharmakokinetics of the photosensitizer based on indotricarbocyanine dye *in vivo*.

**Analysis of intracellular pH and metabolic changes in cancer cells during apoptosis using FLIM-FRET imaging**

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Apoptosis is a physiological process essential for normal tissue development and homeostasis. Activation of caspase-3 has been recognized to play a pivotal role in two major apoptotic pathways. Tumor cells evade apoptosis using different mechanisms. Alterations of intracellular pH (pHi) and energy metabolism are principal features of tumor cells. The aim of this study was to analyze metabolic changes, pHi and caspase-3 activation in cancer cells during apoptosis. We investigated the metabolic shifts in tumor cells based on the fluorescence and fluorescence lifetime of the metabolic co-factors NAD(P)H and FAD. The measurements of pHi and caspase-3 activation were performed using the genetically encoded sensors SypHer1 and mKate2-DEVD-iRFP, respectively. CT26 (murine colon carcinoma) cell lines stably expressing the sensors were used in the experiments. For apoptosis induction, CT26 cells were treated with staurosporine (STS). The onset of apoptosis in CT26 cells was observed after STS treatment. Analysis of pHi in cancer cells using SypHer1 showed the changes during apoptosis. Acidification was found to precede caspase-3 activation in cancer cells undergoing apoptosis in response to STS. Study of fluorescence lifetime in CT26 cells expressing mKate2-DEVD-iRFP revealed an increase in mKate2 fluorescence lifetime, indicating caspase-3 activation after STS treatment. Moreover, a metabolic switch from glycolysis to oxidative phosphorylation was observed in tumor cells during apoptosis - the fluorescence lifetime of NAD(P)H changed from free to bound form at relatively early time points. Therefore, we showed that mechanisms of apoptosis are highly complex and involve an energy dependent cascade of molecular events. Alterations in cellular pH regulation and activation of oxidative phosphorylation represent early events associated with apoptosis induction. Elucidating the molecular targets responsible for cell decision between apoptosis and proliferation is supposed to be the goal of most cancer therapies to overcome resistance of tumor cells to apoptosis. This work was financially supported by the Russian Science Foundation (project # 14-25-00129).

**ADFLIM in AD Research –Imaging Mitochondrial Function in Alzheimer´s Disease**

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Mitochondrial dysfunction is known as an early feature of Alzheimer´s disease (AD). Amyloid beta (Aβ) as well as its precursor protein APPwere identified as key players provoking these mitochondrial disturbances. This entails an energy imbalance in the brain, being one trigger of neuronal death in Alzheimer´s disease.

To further elucidate the role of the intracellular localization of both proteins in mitochondrial impairment, we performed metabolic characterizations of intact cells overexpressing the respective proteins. Using high-resolution respirometry and electron microscopy, we demonstrate especially the intracellular/mitochondrial pool of Aβ to lower mitochondrial respiration.

As the toxic potential of intracellular Aβ underlines the rational of a selective vulnerability of different cell types to Aβ-induced mitochondrial defects, we established a multimodal optical system to measure cell metabolism on the single cell level. Relying on NADH fluorescence lifetime imaging microscopy (NADH FLIM), here we demonstrate that our optical metabolic imaging system is able to mirror the results obtained in the Oroboros Oxygraph-2k and in surplus displays subcellular resolution representing mitochondrial and neuronal heterogeneity in AD.

Our results demonstrate the importance of assessing energy metabolism on the single cell level to shed light onto Alzheimer´s disease associated mitochondrial dysfunction, highlighting the potential of NADH FLIM for metabolic characterization.

**Cellular and subcellular optical imaging in neuroscience**

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Modern methods of optical neuroimaging have substantially increased our ability to investigate signaling and plasticity in the brain. It is now possible to monitor thousands of neurons at work, or reveal Ca2+and voltage dynamics in small cellular compartments. In this talk, I would like to share our experience of using various imaging techniques to study neurons and astrocytes. We use voltage sensitive dyes to monitor propagation of excitability in neuronal networks with fluorescent imaging. We have also explored a possibility of using these dyes for second-harmonic generation (SHG) voltage imaging in small neuronal compartments. However, most of our data have been obtained with Ca2+imaging (widefield, confocal and two-photon), that has been performed in cells either filled with chemical Ca2+ dye or expressing genetically encoded Ca2+sensor (GCaMP2). Optical methods are also used for photostimulation. We routinely use local glutamate uncaging, and explored a possibility of using Channelrhodopsin-2 (ChR2) for subcellular stimulation. I will summarize benefits and caveats of these approaches and discuss what we expect in the near future in optical neuroimaging.

**Analysis of intracellular pH and metabolic changes in cancer cells during apoptosis using FLIM-FRET imaging**

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Apoptosis is a physiological process essential for normal tissue development and homeostasis. Activation of caspase-3 has been recognized to play a pivotal role in two major apoptotic pathways. Tumor cells evade apoptosis using different mechanisms. Alterations of intracellular pH (pHi) and energy metabolism are principal features of tumor cells. The aim of this study was to analyze metabolic changes, pHi and caspase-3 activation in cancer cells during apoptosis. We investigated the metabolic shifts in tumor cells based on the fluorescence and fluorescence lifetime of the metabolic co-factors NAD(P)H and FAD. The measurements of pHi and caspase-3 activation were performed using the genetically encoded sensors SypHer1 and mKate2-DEVD-iRFP, respectively. CT26 (murine colon carcinoma) cell lines stably expressing the sensors were used in the experiments. For apoptosis induction, CT26 cells were treated with staurosporine (STS). The onset of apoptosis in CT26 cells was observed after STS treatment. Analysis of pHi in cancer cells using SypHer1 showed the changes during apoptosis. Acidification was found to precede caspase-3 activation in cancer cells undergoing apoptosis in response to STS. Study of fluorescence lifetime in CT26 cells expressing mKate2-DEVD-iRFP revealed an increase in mKate2 fluorescence lifetime, indicating caspase-3 activation after STS treatment. Moreover, a metabolic switch from glycolysis to oxidative phosphorylation was observed in tumor cells during apoptosis - the fluorescence lifetime of NAD(P)H changed from free to bound form at relatively early time points. Therefore, we showed that mechanisms of apoptosis are highly complex and involve an energy dependent cascade of molecular events. Alterations in cellular pH regulation and activation of oxidative phosphorylation represent early events associated with apoptosis induction. Elucidating the molecular targets responsible for cell decision between apoptosis and proliferation is supposed to be the goal of most cancer therapies to overcome resistance of tumor cells to apoptosis. This work was financially supported by the Russian Science Foundation (project 14-25-00129).

**The studying of oligomeric state of caspase-3 FRET-sensors TR-M5-K and TR-M6-K**

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In our laboratory the caspase-3 sensor TR-M4-K based on TagRFP and KFP fluorescent proteins and a flexible polypeptide linker with the structure of "buoy" was created. It has been demonstrated that the sensor was successfully hydrolyzed by caspase-3 *in vitro* and in living tumor cells. To optimize the structure of the linker variation of the length of its hydrophobic part was conducted and the sensors TR-M5-K and TR-M6-K were created by adding one or two pairs of hydrophobic amino acid residues respectively. Studies of the properties of these sensors showed that unlike the TR-M4-K, which has a tetramer structure, these sensors are in the form of macromolecular aggregates in solution.

Aggregation prevents the correct folding, hinders access to the site of the enzyme hydrolysis and complicates their use in living systems because of the large size of aggregates. In order to prevent aggregation different methods, that either prevent aggregation in the process of protein biosynthesis or destroy protein oligomers which have already formed, are used. The latter methods involve usage of surfactants and chaotropic agents.

For the destruction of protein aggregates of TR-M5-K and TR-M6-K imidazole (0.5M to 3M), Triton X-100 (0.01% to 1%), potassium isothiocyanate (1M to 6M) and guanidinium hydrochloride (0.5M to 6M) were used. The oligomeric state was determined by the method of dynamic light scattering.

The best results on reducing aggregation were obtained using a 0.01% solution of Triton X-100 and TR-M5-K sensor. In this case about 80% of the sensor were present in the octameric form along with the high molecular weight aggregates. Other detergents in various concentrations didn't reduce the oligomeric state of both sensors.

This work was supported by Russian Science Foundation, grant № 15-14-30019.

**Measuring viscosity in cancer using molecular rotors and FLIM**

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Intracellular viscosity is an important parameter regulating membrane permeability, transport, enzyme activity, diffusion controlled functions, biosynthesis, interaction between macromolecules etc. Viscosity in cancer cells and tissue has been very poorly studied to date. It is known that it differs for cancer and normal cells and changes under the therapy, though. Our work was aimed at the development of the method for imaging microviscosity in cancer cells using Bodipy-based rotors and fluorescence lifetime imaging microscopy (FLIM). Two molecular rotors were tested on CT26 murine colon carcinoma in vitro and tumors in mice in vivo. In vitro the cells were incubated with 8.9 mM rotor solution. In vivo the rotors were injected intravenously at the doses 3-7 mg/kg. Multiphoton tomograph MPTflex (JenLab, Germany) with TCSPC-based FLIM module (Becker&Hickl Inc., Germany) was used for registration fluorescence lifetimes. It was shown on the cell culture that Bodipy 2 stains preferentially plasma membrane within 10 min after adding to the plate. The fluorescence decay exhibited monoexponential behavior with ~2.6 ns lifetime that corresponds to viscosity of ~ 370 cP. Pharmacokinetics study showed that concentration of rotors in the blood plasma of tumor-bearing mice decreased exponentially by 48 h after intravenous administration. Based on the fluorescence whole-body imaging in vivo we found that both rotors accumulated in the CT26 tumor with maximum in the period 1-4 h for Bodipy 2, and 6 hours for Bodipy C10 dissolved in polymeric brushes. Two-photon fluorescence microscopy in vivo showed that the rotors are located in cancer cells and connective tissue. However, the rotor located in fibrotic stroma had uncharacteristic fluorescence decay, presumably due to aggregation or interaction with protein, which limited viscosity determination. Measurements of fluorescence lifetimes of the rotors in tumor tissue showed their redistribution between cytoplasm and membrane structures during 1.5 h post-injection. Viscosity values detected in vivo were similar to those measured in cell culture. Therefore, we demonstrate the possibility for in vitro and in vivo viscosity analysis in living cancer cells. The work was financially supported by the RFBR (15-02-05189).

**Bioluminescence Resonance Energy Transfer system based on Firefly *L. mingrelica* Luciferase and its application for the Rapid Homogeneous Immunoassay**

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Bioanalytical systems based on the Bioluminescence Resonance Energy Transfer (BRET) are widely used in fundamental biochemical studies, as well as for screening and analysis of biologically active compounds. The *Renilla* luciferase is the most popular energy donor for BRET. However, its bioluminescence has a low quantum yield and a short-wave peak (480-535 nm) of the spectrum, which complicates *in vivo* imaging in mammalian tissues. Firefly (beetle) luciferases alleviate these drawbacks and show great promise for applications in BRET systems. Different versions of BRET systems based on firefly luciferases are used for highly sensitive determination of proteases and for homogeneous immunoassays.

The sensitive BRET system for the homogeneous immunoassay of a low molecular weight antigen was developed using progesterone as an example. Thermostable mutants of the *Luciola mingrelica* firefly luciferase were tested as the donors whereas the water-soluble Alexa Fluor 610х (AF) dye was selected as the acceptor. The optimal pair donor–acceptor was selected. We developed the methods for the synthesis of the luciferase–progesterone (Luc–Pg) conjugate and the conjugate of the dye and the polyclonal anti-progesterone antibody (AF–Ab). Both conjugates retained their functional properties, had high antigen–antibody binding activity, and demonstrated an efficient BRET. The homogeneous immunoassay system based on the BRET from the firefly luciferase to the synthetic dye was established to assay progesterone as a model antigen. Optimization of the assay conditions, the composition of the reaction mixture, and the concentrations of the donor and the acceptor made it possible to reach the minimum detectable progesterone concentration of 0.5 ng/ml.

**New fast photoconvertible protein SAASoti.**1,2 Solovyev I.D.,3 Vinokurov L.M., 4 Ivashina T.V., 1 Savitsky A.P.

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Photoconvertible proteins are widely used as a label in super-resolution localization microscopy. Application of fast photoconvertible proteins is preferable to decrease light dose that has a toxic effect on cells. SAASoti is a photoconvertible protein obtained from coral *Stylocoeniella armata* (green *Ex* 509 nm/*Em* 519 nm, red *Ex* 579/*Em* 589 nm). Wild type SAASoti is a tetramer with tendency to aggregation. We prepared several mutant forms of the protein and K145E SAASoti variant was observed as tetramer only. Protonated chromophore group is photoconvertible which has 400nm absorbance peak. Therefore photoconversion depends on pK1. We used 395nm 20mW/cm2 led light source to convert proteins. We describe the process as a consecutive reaction, conversion k1 is the first step and bleaching k2 is the second step. Rate constants were normalized on protonated deprotonated form absorbance ratio 400/509nm (velocity(pH) dependence has the same manner). Constants values were measured for: wild type SAASoti (pK1=6.5 k1=0.7 s-1, pK2=5.3 k2=0,1 s-1), K145E (SAASoti) (pK1=6.5 k1=0.7 s-1, pK2=6.3 k2=0,1 s-1) We used commonly used commercial Dendra2 (pK1=7.0 k1=0.03 s-1) for the comarison, Dendra2 photobleaching was not registerеd under these conditions. SAASoti photoconversion rate exceeds dendra2.  
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**Wide-field Time-Correlated Single Photon Counting FLIM**

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Time-correlated single photon counting (TCSPC) is a widely used, sensitive, precise, robust and mature technique to measure photon arrival times in fluorescence spectroscopy and microscopy, light detection and ranging (lidar) and optical tomography. In wide-field TCSPC, the position and the arrival time of the photons are recorded simultaneously, and no scanning is required. Our recent work in this field ranges from microsecond resolution phosphorescence lifetime imaging (PLIM)1-3 to nanosecond fluorescence lifetime imaging (FLIM) microscopy.4 The latter is based on a crossed delay-line anode detector with picosecond timing resolution, where the readout is performed by three standard TCSPC boards.5 This approach retains all the advantages of TCSPC and extends them to wide-field detection, serving essentially as a single photon sensitive camera with picosecond resolution. The operation of the system is fully integrated in the TCSPC acquisition software and allows rapid preview of the acquired images, an essential feature to facilitate focussing, sample positioning and excitation power adjustment. Data is saved as a conventional x,y,t FLIM data cube, and data analysis is performed in the usual way by FLIM analysis software. The data obtained with the system feature good time resolution with an IRF below 230 ps, and reasonably good spatial resolution below 250 μm over the 40 mm diameter detector. We apply the system to FLIM of cells labelled with membrane dyes, imaged with a TIRF microscope using less than 7 mW/cm2 excitation power over the whole field of view. This approach is also ideal for lightsheet FLIM.

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**Differeng stages of fibril formation as seen in intrinsic and extrinsic fluorescence**

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Protein misfolding and its subsequent aggregation is a widely investigated phenomenon as it is responsible for various disorders such as Parkinson and Alzheimer diseases etc. These disorders are associated with the formation and accumulation in brain of compact plaques – highly organized β-sheet-rich amyloid fibrils. Amyloid fibrils share common structural characteristics despite the fact that they can be constructed from absolutely different proteins or peptides. Fibril formation includes such structural stages as native protein state, partially-folded intermediates, formation of protofilament, protofibrils and, finally, mature fibrils [1]. However, the kinetic aspects of these transitions are largely unknown, thus stimulating the development of methods for monitoring different stages of aggregation. Here we investigated the physical and chemical transformations associated with proteins fibrillation (by the example of insulin and lysozyme) by means of its intrinsic and extrinsic fluorescence. We made use of steady state and time-resolved fluorescence spectroscopy using thioflavin T and intrinsic fluorophores to assess different stages of fibril formation. Namely, the changes in Thioflavin T intensity and its lifetime were used to follow prefibrillar structures formation. We also used the intrinsic fluorescence of tyrosine (the main chromophore in insulin) and pi-stacking structures [2] to monitor the initial transformations happening in proteins under fibrillation. Pi-stacking is the nonbonding interaction between aromatic rings in proteins that results in the appearance of new fluorescence band (λex/ λem = 350/430 nm). To visualize directly the ways that protofibrils interact with each other to form mature fibrils, the cryo-electron microscopy (EM) was applied.

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**Tissue and cell optical clearing as a tool for enhaced microscopy and imaging: from *in vitro* to *in vivo***

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Fundamentalsand advances of optical clearing (OC) for enhanced microscopy and imaging of living tissues and cells will be discussed. The OC technology is based on control lable and reversible modification of tissue optical properties by their impregnation by exogenous optical clearing agents (OCAs) [1-3]. Impact of different OCAs on water transport in tissues and temporal tissue optical properties will be analyzed. Tissue reversible dehydration and induced transverse and longitudinal shrinkage measured *in vitro* and *in vivo* will be discussed. The specific features of OC of fibrous and loose connective tissues, as well as epithelial tissues are investigated using OCT, confocal microscopy, photoacoustic microscopy, linear and nonlinear fluorescence and SHG microscopy, and speckle dynamic imaging. Enhancement of probing depth and image contrast in *in vitro*, *ex vivo*, and *in vivo* studies of a variety of human and animal tissues, including skin, fat, eye sclera, muscle, cerebral membrane, digestive tract tissue, cartilage, bone, blood vessels, and blood will be demonstrated. The technologies of effective OCA delivery, including hidden free diffusion, local heating, enforced tissue permeability (sonophoresis, laser perforation), OCA encapsulation, and via blood and lymph vessel networking,will be also discussed. Impact of different OCAs on tissue structure, free/bound water balance and microcirculation will be analyzed. Experimental results on diffusivity of glucose, glycerol, PEG, OmnipaqueTMand other biocompatible clearing agents in normal and pathological tissues will be presented.

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**Fluorescence visualization of the tumours of the laboratory animals with the using genetic encoded sensors.**

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The stable transfection of cancer cells with fluorescent protein (FP) genes opens up the possibility for highly specific genetic tumor labelling. It is important that the cancer cells express the FP in a desirable cell compartment, throughout their whole life, and that this ability is passed from one generation to the next. Therefore, such genetic marking offers new opportunities for solving a variety of problems ranging from basic studies of the process of carcinogenesis to real-time investigations for drug-response evaluation.

Fluorescence diffuse tomography (FDT) is the most accurate technique for the imaging of labeled tumors in the small animal body. But the procedure for reconstruction of the spatial distribution of the fluorophore requires a high signal-to-noise ratio due to the ill-condition of the inverse problem. Therefore, the FDT technique is ineffective for imaging tumors of small size or with dim fluorophores, because of the low intensity of their fluorescence compared with the high level of tissue autofluorescence. In these cases, the size and position of a marked tumor in the animal body can be estimated from 2D fluorescent images obtained using trans- or epi-illumination techniques.

We have created and tested a versatile system for small animal fluorescence imaging which combines planar epi- and trans-illumination geometries of the light source and of the fluorescence receiver. For epi-illumination we use homogeneous illumination of the experimental animal, in combination with a CCD camera which covers over the illuminated area. For trans-illumination we use mechanical raster-scanning devices for the laser source, modulated at a low frequency, and a cooled photomultiplier tube which provides outstanding sensitivity. The monitoring which we have conducted of orthotopic tumorgrowth in animal bodies has demonstrated the efficacy of trans-illumination imaging in comparison with the epi-illumination technique.

**Firefly luciferase as a probe for imaging and monitoring in living systems**

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Optical reporters are widely used in cell biology to tag and monitor molecular processes non-invasively in living cells or in whole intact animals. While less bright than fluorescent probes, bioluminescent systems have several distinct advantages since they do not require external illumination and have extremely low background signals. The applications of luciferase reporters fall into two main groups. Firstly, bioluminescence can be used to monitor temporal changes in gene expression and protein-protein interactions. Secondly, specific types of cells can be marked with constitutively high levels of luciferase and then imaged in intact animals to follow tumor growth or spread of pathogens. One can monitor either a total light output from an object with a photomultiplier tube or perform a continuous 2D-imaging with a highly sensitive camera. The particular benefit of the beetle luciferin/luciferase system is the ability to perform continuous long-term real-time monitoring both in live cell cultures and in animals up to several weeks and with up to single cell resolution.

We report novel mutants of *Photinus pyralis* firefly luciferase that outperform the widely used beetle luciferases (Eluc, luc2) in brightness in live mammalian cells. Furthermore, some mutants provide faster response to transcriptional changes (surpassing the efficiency of the CL1-PEST degradation tag in reducing luciferase half-live). Interestingly, the shorter half-life of the latter mutants is mediated by a real-time inhibition of luciferase activity in the course of the recording and not due the protein degradation. Thus, their fast performance is expected to be less dependent on the state of proteasomal machinery. Of note, beetle luciferases from different species show widely different protein and mRNA half-lives (associated with their protein and coding DNA sequences). For example, the mutant LmGTS of *Luciola mingrelica* luciferase provides a faster response as inducible reporter due to its short-lived mRNA and relatively short protein half-life compared to short-lived variants of *P. pyralis* luciferase. Our findings promote the design of superior beetle luciferase reporters for bioluminescence imaging and monitoring in living systems.

**Super-resolution fluorescence microscopy for investigation of bacterial cytoskeleton**

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Bacteria sizes are comparable to diffraction limit of conventional fluorescence microscopy, thus super-resolution fluorescence microscopy (which is able to overcome this limit) seems to be very attractive tool to study internal organization of bacteria. Single-molecule localization microscopy (SMLM) is a powerful fluorescence microscopy technique, providing spatial resolution which is far beyond the diffraction limit. SMLM appears to be a flexible tool to study bacterial internal structures, including arrangement of proteins in bacterial divisome. One of divisome proteins isa prokaryotic tubulin homologue FtsZ which plays a key role in cell division, forming the Z-ring between dividing cells[[1](#_ENREF_1)]. Using SMLM we have shown that Z-ring thickens during constriction [[2](#_ENREF_2)]. Furthermore, this approach was used to investigate organization of one of the smallest bacteria - *Acholeplasma laidlawii,* which size (about 0.5 μm in diameter) does not allow to resolve internal structures using conventional fluorescence*.* To visualize IbpA (small heat-shock protein) structures in *A. laidlawii*we utilized SMLM in combination with immunofluorescence staining which allows to work with these small microorganisms for which effective genetic manipulation protocols haven’t yet been developed. Also this method provides better resolution than widely utilized fluorescent fusion proteins approach. This technique allowed us to obtain images of some IbpA distribution patterns in different conditions. We were not able to resolve these patterns using conventional fluorescence microscopy, and this fact emphasizes the power of SMLM. Furthermore, it encourages to expand utilization of super-resolution methods by applying them to other, less studied organisms.

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**In-vivo time-domain diffuse optical imaging of the adult human brain**

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When near-infrared light enters the human head, it is strongly scattered and propagates in a nearly diffusion-like manner through the tissue. Recording light that exits the head up to several centimeters apart from the point of injection allows one to study the absorption and scattering properties and their changes inside the tissue volume. Due to the strong scattering, spatial resolution is restricted to ~1 cm. The reflectance geometry is the only way to perform optical spectroscopy and imaging of the brain in adults. In this geometry, picosecond time resolution adds substantial information to the measurement since the average penetration depth of photons is related to their total time of flight. In particular, absorption changes occurring in the brain and in the skin can be separated. Our group pursued two approaches to time-domain optical brain imaging, (1) mapping at multiple (up to 16) sites on the head by fiber-based optodes attached to the scalp with a separation of typically 3 cm, (2) non-contact scanning that provides a dense and flexible grid of, e.g., 32x32 measurement positions that cover an area extended over several centimeters while the source-detector separation is a few millimeters only. In this case, a fast-gated single-photon avalanche diode is employed to sensitively detect late photons while eliminating the huge amount of early photons that do not carry information about deep tissue compartments, in particular the brain. In both approaches, short laser pulses are injected and photon time-of-flight distributions are recorded by time-correlated single photon counting. We developed various methods to separate cerebral (deep) and systemic (superficial) contributions to the signals, based on the analysis of time windows or statistical moments of the time-of-flight distributions. Technical details of the related instrumentation as well as of the data analysis, advantages and limitations of both approaches will be presented. The major in-vivo application of the technique is the study of functional brain activation, in particular by motor or cognitive stimulation, that is accompanied by changes in the concentrations of oxy- and deoxyhemoglobin. Moreover, cerebral perfusion can be assessed by bolus tracking of the contrast agent indocyanine green.

**Advanced quantitative biomolecular analytics in free solution.**

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Interactions Analysis and beyond

MicroScale Thermophoresis (MST) is a powerful technique to quantify biomolecular interactions. It is based on thermophoresis, the directed movement of molecules in a temperature gradient, which strongly depends on a variety of molecular properties such as size, charge, hydration shell or conformation. Thus, this technique is highly sensitive to virtually any change in molecular properties, allowing for a precise quantification of molecular events independent of the size or nature of the investigated specimen.

When performing a MST experiment, a temperature gradient is induced by an infrared laser. The directed movement of molecules through the temperature gradient is detected and quantified using either covalently attached or intrinsic fluorophores. By combining the precision of fluorescence detection with the variability and sensitivity of thermophoresis, MST provides a flexible, robust and fast way to dissect molecular interactions.

Here, we present recent progress and developments in MST technology and focus on MST applications beyond standard biomolecular interaction studies. By using different model systems, we introduce alternative MST applications - such as determination of binding stoichiometries and binding modes, analysis of protein unfolding, thermodynamics and enzyme kinetics - and also demonstrate the capability of MST to quantify high-affinity interactions with dissociation constants (Kds) in the low picomolar (pM) range as well as protein-protein interactions in pure mammalian cell lysates.

Easy and Rapid Analysis of Protein Stability by nanoDSF

nanoDSF is an advanced Differential Scanning Fluorimetry technology. It detects smallest changes in the fluorescence of tryptophan present in virtually all proteins.

The fluorescence of tryptophans in a protein is strongly dependent on its close surroundings. By following changes in fluorescence, chemical and thermal stability can be assessed in a truly label-free fashion. The dual-UV technology by NanoTemper allows for rapid fluorescence detection, providing an unmatched scanning speed and data point density. This yields an ultra-high resolution unfolding curves which allow for detection of even minute unfolding signals. Furthermore, since no secondary reporter fluorophores are required as in conventional DSF, protein solutions can be analyzed independent of buffer compositions, and over a concentration range of 250 mg/ml down to 5 μg/ml. Therefore, nanoDSF is the method of choice for easy, rapid and accurate analysis of protein folding and stability, with applications in membrane protein research, protein engineering, formulation development and quality control.

Here we present biophysical background behind the Prometheus NT.48 instrument and examples of different applications in both academic and industrial context.

**Fluorescence fluctuation and super-resolution techniques - fundamental biomolecular studies and towards clinical diagnostics**

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In this presentation it will first be presented how long-lived, non-fluorescent, photo-induced transient states of organic fluorophores and their dynamics can provide additional, to-date largely unexploited, information about biomolecules, their interactions and their immediate environment. By two major approaches, where the transient state information is obtained either from fluorescence fluctuation analysis or by recording the time-averaged fluorescence response to a time-modulated excitation, it is possible to combine the detection sensitivity of the fluorescence signal with the environmental sensitivity of the long-lived transient states [1,2]. Proof-of-principle experiments, advantages, limitations and applications will be discussed, including live cell transient state (TRAST) imaging of cell membrane fluidity and cellular metabolism [3,4].

Second, it will be shown how diffraction-unlimited imaging of cellular protein distribution patterns using Stimulated Emission Depletion (STED) nanoscopy can potentially provide new diagnostic parameters on the level of individual cells, and also give further insights into underlying disease mechanisms [5,6]. Examples including cultured cells, clinically sampled breast cancer cells and platelets will be given.

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**Tumor metabolism: fluorescence imaging with autofluorophors and genetically encoded sensors**

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We report here on some results about the specific tumor cells parameters obtained by the fluorescence imaging: one-photon and two-photon microscopy, FLIM, STORM and fluorescence whole-body imaging. A few parameters that potentially can change as a result of cancer transformation and anticancer treatment were studied – intracellular pH (pHi), hydrogen peroxide level, metabolic status. The study was performed on monolayer cell cultures, co-cultures of human cancer cells and fibroblasts, tumor spheroids and tumor xenografts.

New genetically encoded ratiometric biosensors SypHer2 [1] and HyPer2 [2] based on the fluorescent protein cpYFP were used to detect pHi and hydrogen peroxide, correspondingly. Cell metabolism was analyzed by NADH and FAD fluorescence lifetime and calculated optical redox ratio FAD/NAD(P)H.

The method of pHi mapping in tumor spheroids and tumors in vivo was developed. More acidic pHi in the core of the tumor nodule was demonstrated, that may be a consequence of hypoxia-induced glycolytic metabolism [3].

Metabolic imaging in living cells showed the differences in a cell metabolism between cancer cells and fibroblasts. As expected, in co-culture conditions human cancer cells switched their metabolism to glycolysis, similar to real tumors. Slight acidification of the cytoplasm of cancer cells was detected in the co-culture, whereas production of hydrogen peroxide increased significantly. These findings testify to the important role of hydrogen peroxide in cellular interactions and metabolic cooperation of cancer cells and fibroblasts for supporting carcinogenesis [4].

Also we made a tracing of the early and delayed alterations in cancer cells metabolism and pHi level under the influence of chemotherapeutic drugs with different mechanism of action: cytotoxic agent Cisplatin and cytostatic agent Taxol. Acidification was an early answer after incubation with Cisplatin, which is caused by fast inhibition of Na+/H+ membrane exchanger-1, then pHi dynamic pattern in survived cells was characterized by long stable alkalization period. At the first hour after Taxol treatment we observed rapid cancer cells cytosol alkalization that are corresponding to the maximum of Taxol uptake. Then pHi level decreased and fluctuated near the initial value up to the timepoint 24 hours. Optical redox ratio and Fluorescence lifetimes of NAD(P)H illustrated change of metabolic states tumor cells after treatment to more Oxidative phosphorylation.

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**FLIM-FRET of genetically encoded sensor of caspase 3 in tumor xenografts**

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Noninvasive monitoring of molecular events *in vivo* is one of the paradigms of molecular fluorescence imaging. In our investigation the FLIM-FRET of caspase 3 based on TR23K genetically encoded sensor in tumor subcutaneous xenografts was demonstrated. We used the FRET-pair containing the TagRFP as a donor and KFP as acceptor linked by 23a.a. linker including the caspase site specific motif DEVD (TR23K sensor [1, 2]) for imaging of caspase 3 in intact tumor xenografts and in tumor xenografts after the treatment. The confocal scanning system DCS-120 (Becker & Hickl GmbH, Германия) was used for the FLIM read out. The shift in lifetime distribution from 1.6-1.9 ns to 2.1-2.4 ns after injection of paclitaxel to A549 lung adenocarcinoma xenograft, etoposide and cisplatin to HEp-2 pharynx adenocarcinoma xenograft was observed. The FRET efficiency and antitumor therapy efficiency was estimated using SPCM software (Becker & Hickl GmbH, Germany). The different depth of tumor invasion, heterogeneity in tumor response as well as spontaneous apoptosis in tumors impact on distribution of lifetimes *in vivo*.

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