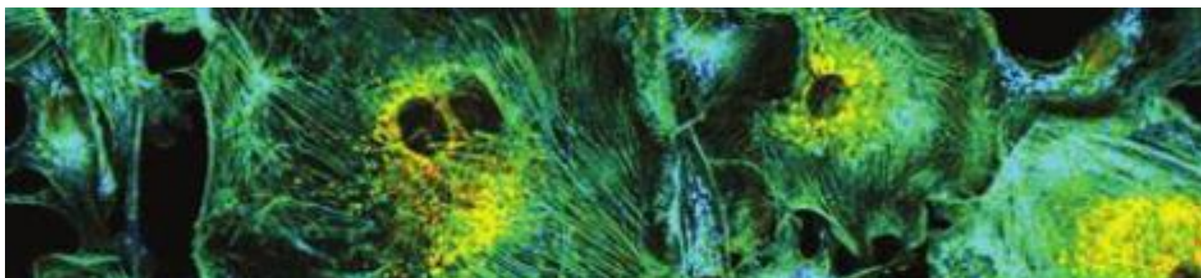

e- Book of Abstracts of the 3rd School on Advanced Fluorescence Imaging Methods (ADFLIM)

Saratov State University
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Plenary Sessions

The Multi-Dimensional World of TCSPC FLIM

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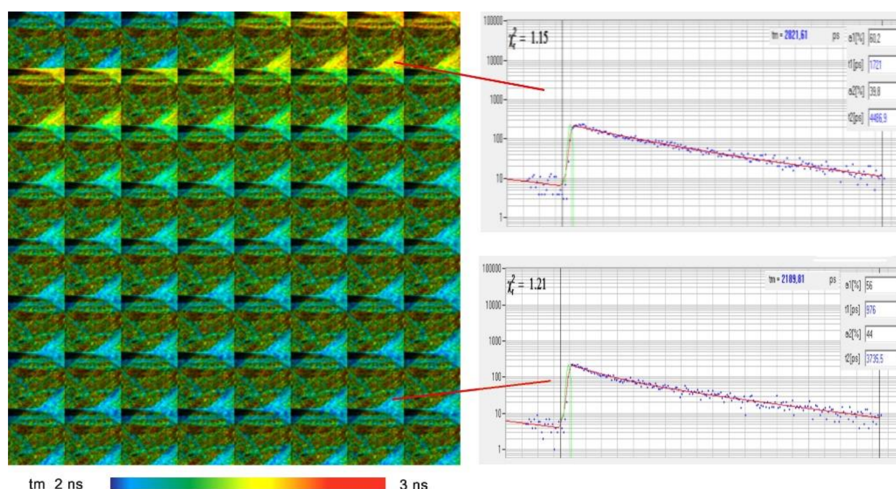
TCSPC FLIM delivers a photon distribution over the image coordinates and the time after the excitation pulses. The technique not only reaches an extraordinary time resolution and photon efficiency, it also records the decay data in the individual pixels into a large number of time channels.

Often the decay data in the pixels are simply parameterised by a single 'decay time'. However, this misses the point of TCSPC FLIM. TCSPC FLIM data are inherently multi-dimensional in a multi-dimensional parameter space. The decay functions in the individual pixels usually contain several exponential components, and are characterised by several independent decay times and amplitude factors. These are directly related to biological information. Examples are FRET experiments, where the decay functions represent an interacting and a non-interacting donor fraction, and metabolic imaging, where the components represent bound and unbound NADH.

An entirely new level of complexity is reached by extending the photon distribution of TCSPC FLIM itself by additional parameters. Examples of such parameters are the wavelength of the photons, the depth of the focus in the sample, the time after a stimulation of the sample, or the time within the period of an additional modulation of the laser. The corresponding photon distributions can be four- or five-dimensional, the data representing multi-spectral FLIM, FLIM Z stacks and lateral FLIM mosaics, time-series FLIM, and simultaneous FLIM-PLIM, or even combinations of these. Possible applications are the investigation of fast physiological effects in live systems, such as Ca^{++} transients or chlorophyll transients, dynamics of protein interaction, and metabolic imaging in combination with pO2 measurement. There may be more which have not even been considered yet. This paper is an attempt to spread knowledge about advanced TCSPC techniques to potential users and start a discussion about future applications.



Example: Calcium transient in live neurons, recorded by multi-dimensional TCSPC. Time resolution 40 ms per image

Fast-Acquisition TCSPC FLIM with sub-25 ps IRF Width

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There is currently a run towards faster and faster acquisition of Fluorescence Lifetime Imaging (FLIM) data. Fast-FLIM techniques normally use time-gating into only a few time windows, or a multichannel scaler process with a time-channel width in the 200-ps range or longer. The techniques are often combined with wide-field detection to obtain parallel acquisition in all pixels of the image. Compared with TCSPC FLIM, the time resolution, both in terms of IRF width and time channel width, is lower, and the ability to resolve multi-exponential decay profiles into their components is limited. However, in typical FLIM applications, such as FRET imaging or metabolic imaging, exactly these features are important. Moreover, wide-field detection does not deliver optical sectioning and does not suppress lateral scattering.

It is commonly accepted that TCSPC FLIM delivers the best time-resolution and the best photon efficiency of all FLIM techniques. TCSPC FLIM data are virtually free of lateral scattering and out-of focus signals. The technique is also able to record the data into a sufficient number of sufficiently small time channels so that multi-exponential decay analysis is possible. There are also other advantages, such as the capability to record multi-wavelength data, simultaneous FLIM and PLIM, and extremely fast triggered time series. It therefore appears unwise to discard the TCSPC technique until all options of increasing the recording speed have been exploited.

We will show that the commonly used bh TCSPC FLIM devices are able to record and display lifetime image at a rate of several images per second. To further increase the acquisition speed we present a FLIM system comprising a single detector, four parallel TCSPC channels, and a device that distributes the photon pulses into the four recording channels. The system features an electrical IRF width of less than 7 ps (FWHM), and a time channel width down to 820 fs. The optical IRF with an HPM-100-06 hybrid detector is shorter than 25 ps (FWHM). The system is virtually free of pile-up effects and has drastically reduced counting loss. FLIM data can be recorded at acquisition times down to the fastest frame times of the commonly used galvanometer scanners. Fast recording does not compromise the time resolution; the data can be recorded with the TCSPC-typical number of time-channels numbers of up to 1024 or even 4096. Pixel numbers can be increased to 1024 x 1024 or 2048 x 2048 pixels. The system is therefore equally suitable for fast FLIM and precision FLIM applications.

Quantitative single-molecule FRET measurements of F_oF_1-ATP synthase at work in an ABELtrap

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Conformational changes of single proteins can be monitored in real time by Förster resonance energy transfer, FRET. Two different fluorophores have to be attached to those protein domains which move during function. Distance fluctuations between the fluorophores are measured by relative fluorescence intensity changes or fluorescence lifetime changes.

Accordingly the dynamics of single-molecule FRET fluctuations report the catalytic activity of proteins. We use time-resolved FRET measurements to study individual F_oF_1-ATP synthases in liposomes driven by ATP hydrolysis. Fluorescence anisotropy of FRET donor and acceptor are recorded simultaneously. However, observation times of freely diffusing proteoliposomes are limited by Brownian motion. To counteract this motion actively, we have built a fast anti-Brownian electrokinetic trap (ABELtrap, invented by A. E. Cohen and W. E. Moerner) with a laser focus pattern generated by electro-optical beam deflectors and controlled by a programmable FPGA chip. We demonstrate how Michaelis-Menten kinetics of F_oF_1-ATP synthases can be extracted from single-molecule FRET traces of individual membrane enzymes hold in the ABEL trap.

Determination of intracellular chloride concentration in neuronal tissue by fluorescence lifetime microscopy

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Fluorescence lifetime imaging (FLIM) based on time-correlated single photon counting is a well-established technique in cell physiology imaging, e.g. for investigating metabolism, protein-protein interactions or intracellular ion concentrations. Thedetermination of intracellular chloride concentrations in brain cells and tissue has revealed a number of important details of physiological processes in several astrocyteand neuron types. Here an overview – both on the technical details and improvements
as well as the most relevant results will be given.

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Advanced imaging techniques for surveying the interactions and dynamics of viral and bacterial 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. Single-molecule super-resolution techniques such as PALM, STORM, and PAINT provide a unique opportunity to localize and map individual molecules and perfectly complement the dynamic information. We used these tools to monitor the intracellular fate of the nucleocapsid protein (NCp7) of the Human immunodeficiency virus type 1 (HIV-1). We evidence that the NCp7 molecules are released from viral complexes during their trafficking towards the nucleus, in a mechanism closely related to reverse transcription and capsid disassembly processes. We also showed that the released NCp7 localize mainly in the cytoplasm and the nucleoli, where they bind to ribosomal RNAs. Furthermore, super-resolution PALM/dSTORM helped us defining the distribution of NCp7 molecules in the nucleoli compartments. Besides this viral exemple, we will also present single-molecule tracking of PvdA, a cytoplasmic protein of the Pyoverdin (PVD) biosynthetic pathway in *Pseudomonas aeruginosa*. Detailed analysis tracking data led us to the identify dynamic interactions of PvdA with Non-Ribosomal Peptide Synthetases involved in PVD biosynthesis. Finally, we implemented sPAINT (spectrally-resolved Points Accumulation for Imaging in Nanoscale Topography), a multi-dimensional super-resolution imaging technique to record both the position and spectrum of single fluorescent emitters simultaneously and make hydrophobicity mapping of biological constructs at the mesoscale.

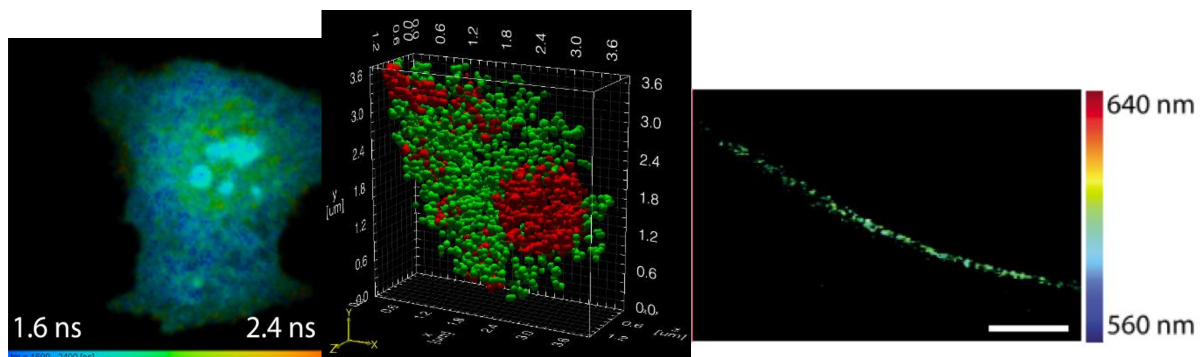

Figure : FRET-FLIM image showing the interaction of HIV-1 NCp7 with nucleic acids (left); two-colour superresolution 3D image of the granular and dense fibrillar sub-domains of the nucleolus (middle), and sPAINT image of SH-SY5Y cells labeled by Nile red (right).

Флуоресцентная микроскопия одиночных частиц и ее применения к изучению структуры и взаимодействий нуклеосом

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В докладе будут рассмотрены принципы флуоресцентной микроскопии на основе Фёрстеровского резонансного переноса энергии для изучения одиночных свободно диффундирующих молекул. Возможности метода будут проиллюстрированы на примере исследований структуры мононуклеосом и их взаимодействий с некоторыми ядерными белками.

Fluorescence Life Time Imaging of tumor metabolism: from cells to patients

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We report here on some results about the specific tumor metabolism obtained by the time-resolved techniques (FLIM microscopy, FLIM and PLIM spectroscopy, FLIM in vivo imaging system). Cancer cell metabolism was analyzed by NADH and FAD fluorescence lifetimes. The study was performed on monolayer cell cultures, tumor spheroids, tumor xenografts and patients. Metabolic changes were used for discovery of new mechanisms of chemotherapy, for analysis of tumor heterogeneity during treatment, as a predictive marker for treatment response and as a specific feature of cancer transformation for diagnosis in patients.

For cell cultures, spheroids and tissue slices the LSM 880 (Carl Zeiss) with FLIM system (Becker & Hickle) was used. The MPT Flex (JenLab) was applied for *in vivo* investigation of metabolic changes in animal models and patients. We showed that metabolic parameters dramatically changed in the process of anticancer treatment and revealed responded and nonresponded cancer cells.

It has been shown, that in patients with skin neoplasia using of metabolic imaging possible to distinguish benign and malignant lesions, such as nevus, basal cell carcinoma and melanoma.

The experimental fiber-optic setup and DCS-120 MACRO. Confocal Scanning FLIM System for Macroscopic Objects. (Becker & Hickle) for simultaneous FLIM/PLIM *in vivo* measurements was used to analyze metabolic and oxygen state of tumor tissue. We proposed a protocol for FLIM/PLIM spectroscopy in one point of tissue with recently reported phosphorescent dye BTPDM1 (Gunma University, Japan) allowing us to reveal a differences between normal and tumor tissues. Also, specific changes of metabolic activity and oxygen state have been shown in vivo after chemotherapy.

Poster Session

Fluorescence microscopy of mammalian oocytes after vitrification

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The aim of the study was to study the viability of mammalian oocytes after vitrification using fluorescence microscopy. The objective of this study was to compare the efficacy of the vitrification of bovine COCs by analysis of morphology and viability after warming. In the study were developed an optimal vitrification technology for the preservation and improve the survival of bovine cumulus-oocyte complexes (COCs). Freezing of oocytes was carried out by vitrification. Morphological assessment of oocytes and fluorescence microscopy were performed after the warming procedure. Two special kits for died/apoptosis cell (PI/Alexa Fluor 488 Annexin, Molecular Probes, USA) and LIVE/DEAD® Viability Assays (Calcein AM (CAM)/ethidium homodimer-1(EtHd-1), Molecular Probes, USA) were used in research for the staining of COCs by instruction. Fluorescence microscopy of 32 bovine oocytes after vitrification and thawing showed that when PI was stained, the proportion of cells was 31.25% (5/16). When stained with apoptosis, the proportion of cells was 25% (4/16). It was also shown that when stained with CAM, the proportion of cells was 87,5 % (14/16). As a result of staining of COCs with fluorescent dyes, it was found that to assess the survival of oocytes should use fluorescent dye CAM. Since fluorescent dyes PI and EtHd-1 did not display the process of cell death of the oocyte. In this regard, PI and EtHd-1 can't act as indicators of the true death of oocytes.

Acknowledgments
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Excimer-forming pyrene pair as a highly efficient FRET-donor within oligonucleotide probes

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We proposed excimer-forming pyrene pair as a fluorescent donor for sCy3. Resonance energy transfer with high efficacy and manifold increase of sCy3 fluorescence were achieved. We applied multi-pyrene label/sCy3 as a D/A pair in oligonucleotide hybridization probes for fluorescent visualization of intracellular long non-coding RNA.

Glutamate-induced [Ca2+]i and mitochondrial potential changes in cultured neurons following an in vitro neurotrauma

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Glu-induced [Ca2+]i and mitochondrial potential changes were measured in rat cultured cortical neurons in 5 min after scratch and also 3 and 7 days following this damage. Mechanical trauma enhanced Glu excitotoxicity and diminished recovery of [Ca2+]i homeostasis and mitochondrial potential after Glu removal.

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New markers of neuronal activity in cognitive tasks and emotional empathy of mice.

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Potential molecular biomarkers of brain plasticity were investigated in mice after fear conditioning training and in empathic fear response. Expression of Pdk4, Leo1 and Nfe2l, Gadd45G as well as classical marker of neuroplasticity c-Fos were analyzed by immunohistochemistry in mice primary cingulate cortex, structure known to be critical for fear learning and empathy. Density of Pdk4, Leo1 and Nfe2l1 immunopositive cells substantially elevated after fear conditioning compared to home cage group. It was shown that Nfe2l1 and c-Fos expression increased in structures associated with emotional empathy in mice.

Expression of HDAC1, HDAC2 and HDAC4 in acute phase after photothrombotic stroke in the rat brain

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We studied the expression of HDACs at 1, 4 and 24 hours after photothrombotic stroke (PTS) in the rat somatosensory cortex using immunohistofluorescence and western-blot analysis. The unilateral irradiation of the somatosensory cortex was performed through the cranial bone using 532 nm diode laser (60 mW/cm2, Ø 3 mm, 30 min). We studied the level of HDAC 1, 2 and 4 in the penumbra and the contralateral hemisphere of animals.

Immunohistofluorescence analysis showed the significant up-regulation of HDAC-1 in the ischemic penumbra at 4 (+105%) but not 1 h or 24 h after PTS. The level of HDAC2 changed more significantly. An increase in protein expression was observed in 4 (+ 86%) and 24 hours (+ 76%), but not 1 hour after the PTS. Both proteins were expressed both in neurons and in the astrocytic processes. These western blots analysis generally validate these results. Western blot analysis and immunofluorescence analysis did not reveal any changes in the HDAC4 content in the brain regions under study at different times after the PTS.

Thus, each HDAC isoform had a specific pattern of expression at different periods after the ischemia. It is known that nonselective inhibitors of histone deacetylases (HDAC) can protect brain cells during ischemia and stroke. Our research indicate that HDAC1 and HDAC2 can be

potential mediators in the acute phase after stroke, suggesting that selective inhibitors of HDAC1 and 2 can be considered for therapeutic approaches in this period.

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Multiphoton microcopy for the investigation of learning and memory mechanisms

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This study addresses t current methods of in vivo two-photon imaging of the activity of neurons involved in episodes of cognitive activity in animals. The principles of fluorescent two-photon microscopy are described and methods for in vivo imaging of neuron activity using calcium indicators of two types – calcium stains and genetically encoded calcium indictors (GECI) – are discussed. A new approach is also considered, using in vivo imaging of genomic activation of cerebral neurons in transgenic animals with fluorescent probes for the expression of the immediate early genes c-fos, Arc, and Egr-1. Moreover, in current study we describe the new approach to investigate the calcium activity of engram neurons, that are involved into the memory coding. We present new Fos-Cre-GCaMP transgenic mice to investigate long-term changes of calcium dynamic in memory engram neurons captured with TRAP technique during training. After genetic recombination and expression of GCaMP, we performed two-photon imaging of calcium fluorescence in the body and dendritic spines of such neurons in cortex during memory recall. Finally, in present study we describe a novel learning task in the Mobile Home Cage setup directly during two-photon imaging.

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The relationship between the activation of caspase-3, metabolic cofactors and cytoplasmic pH in the induction of apoptosis by staurosporin

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A complex cascade of molecular events occurs in apoptotic cells but cell-to-cell variability strongly complicates determination of the order and interconnection between different processes. In this paper we used multiparameter fluorescence microscopy to analyze energy metabolism, intracellular pH and caspase-3 activation in living cancer cells *in vitro* and *in vivo* during staurosporine-induced apoptosis. We performed metabolic imaging of co-factor NAD(P)H, and used genetically encoded pH-indicator SypHer1 and FRET-based sensor for caspase-3 activity mKate2-DEVD-iRFP to visualize these parameters by confocal fluorescence microscopy and two-photon fluorescence lifetime imaging microscopy. We also imaged

endogenous metabolic cofactor NAD(P)H and genetically encoded fluorescent protein mKate2 in a tumor BalbC mice using Macro-FLIM system.
Activation of caspase-3 in the cells was detected before emergence morphologically evidence of apoptosis. The mean fluorescence lifetime (τm) of a donor protein mKate2 in the cells was 1.58 ± 0.11 ns and as result, loss of FRET reaction led to significant increase in mKate2 fluorescence lifetime to 2.32 ± 0.25 ns.

NAD(P)H is known to be a key coenzyme in glycolysis and OXPHOS. The fluorescence decay curves for NAD(P)H were best fit to a double-exponential decay model, indicating the presence of two distinctly different lifetimes for the free and protein-bound forms of this coenzyme. The fluorescence lifetimes of the free (τ1) and protein-bound (τ2) NAD(P)H in the untreated CT26 cells were correspond to data in the literature. No significant changes in the fluorescence lifetime of free form were detected after STS treatment, whereas fluorescence lifetime of protein-bound NAD(P)H increased to 4.07 ± 0.05 ns in 15 minutes after STS treatment and remained at that elevated level during apoptosis.

Analysis of the ratios of the relative contributions of the free to protein-bound NAD(P)H (a1/a2) in the tumor cells showed a decrease in the a1/a2 ratio starting from 15 minutes after STS treatment. The observed changes in the fluorescence lifetimes of protein-bound NAD(P)H and relative contribution of free NAD(P)H testifies a switch toward an OXPHOS.
pHi in CT26 cells was found to be 7.60 ± 0.09 under standard cultivation conditions. This result corresponds to pHi values for tumor cells in the literature.

Treatment of CT26 cells with STS resulted in pHi changes in 15 minutes after exposure. At this time-point the majority of cells (~62%) showed a reduction in pHi compared to the initial value. In 1 hour after exposure to STS a pHi decreased by 0.3-0.4, indicating that acidification might be a common and relatively early event in apoptosis before clear morphological changes appearance. The lower pHi remained in the cells until 24 hours, when shrinkage and blebbing in most cells were detected.

These results indicate that intracellular acidification precedes caspase-3 activation in cancer cells undergoing apoptosis in response to STS. Therefore, cytosolic acidification is supposed to be caspase-dependent.

While FLIM is a powerful approach for cell biology, its small field of view (typically less than 1 mm) makes it impractical for imaging of the larger areas of tissue required for many preclinical and clinical applications. We imaged endogenous metabolic cofactor NAD(P)H and genetically encoded fluorescent protein mKate2 in a mouse tumor using Macro-FLIM system Balb/c mice of 20–22 g body weight were injected subcutaneously in the left flank with CT26 cells (5 × 105 in 200 µL PBS) stably expressing mKate2-DEVD-iRFP sensor. FLIM was performed in vivo on the 14th day of tumor growth, when the tumor size was ∼7 mm× 8 mm.
It is now evident that tumors are metabolically heterogeneous and flexible. The observed values in the fluorescence lifetimes of free and protein-bound NAD(P)H and ratios of the relative contributions comparable to the data shown *in vitro.* The change in the fluorescence lifetime mKate2 was also shown in a mouse tumor using Macro-FLIM system from 2 h after STS treatment.

Experiments are planned to study the pH of the tumor *in vivo.*

Induction of apoptosis was accompanied by the switch to oxidative phosphorylation, cytosol acidification and caspase-3 activation in cell culture.

This is the first time that we detected fluorescence of NAD(P)H and caspase-3 activation across a whole tumor in vivo using macro-FLIM.

This work was partly supported by Russian Science Foundation (grant No14-25-00129)

Oligomerization state of V127T SAASoti fluorescent protein at high concentrations

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Photoconvertible fluorescent proteins (PCFPs) are widely used as markers for the visualization of intracellular processes and for sub-diffraction single-molecule localization microscopy. We have recently purified a novel GFP-like protein, SAASoti, from the coral Stylocoeniella armata. We demonstrated that it is photoconvertible from green to red fluorescence under 405 nm illumination. Under 470 nm illumination green fluorescence of SAASoti and its V127T variant are reversible photobleached without photoconversion to the red form [1, 2]. The phenomenon can be explained by chromophore protonation that was confirmed by increase in absorption at 400 nm (chromophore protonated form) and decrease at 509 nm (anionic form). This photo-induced photoswitching can be repeated several times with the same sample without previous fluorescence intensity loss.

Although wild type of a new photoconvertible fluorescent protein SAASoti tends to aggregate, we succeeded, via rational mutagenesis, to obtain V127T monomeric variant. Monomeric form formation was confirmed either by gel-filtration chromatography or by FCS measurements. Nevertheless, working with V127T SAASoti at high concentrations is accomplished with its partial oligomerization that is the main problem when obtaining crystals, e.g. Addition of reducing agent dithiothreitol (DTT, 10 mM) to the sample and to the system (buffer) leads to disappearance of partially dimerized SAASoti fractions at the concentration of 0.22 mM. V127T SAASoti partial dimerization at high concentrations (0.22 mM) is most likely caused by S-S bond formation between the subunits. After DTT was removed from eluted fractions by dialyzing against 20 mM Tris-HCl pH 7.4, 150 mM NaCl and V127T SAASoti was concentrated we obtained 0.1 mM fraction. Repeated gel-filtration chromatography at non-reductive conditions revealed monomeric state of the protein. Structure analysis of SAASoti model revealed two surface-facing cysteine residues – C21 and C117. Interestingly, that wild type SAASoti still forms stable oligomers under reductive conditions. Thus, one can conclude that V127T mutation disturbs strong a.a. interactions of another chemical nature between the subunits in dimers and tetramers. Structure alignment with other FPs revealed that asparagine occupies the 21 position (SAASoti numbering) in most of the cases. Site-directed mutagenesis (C21N) will help us to test the hypothesis.

This work was supported by the Ministry of Education and Science of the Russian Federation (project 14.W03.31.0023).

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Hyaluronic acid as enhancer for human skin epidermis optical clearing by PEG-300: OCT *in vivo* study

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The use of optical clearing agents makes it possible to increase the probing depth of non-invasive optical diagnostic methods including fluorescent ones. However, the protective epidermal barrier of the skin prevents the penetration of hydrophilic immersion liquids into the deeper layers of the skin. The aim of this study is to increase the efficiency of skin optical clearing by increasing the permeability of the epidermis for polyethylene glycol (PEG-300), which is a biocompatible immersion agent. To increase the permeability of the epidermis, a solution of hyaluronic acid (HA) and sonophoresis were used. The experiments were carried out in vivo with human skin, using an optical coherent tomograph with a working wavelength of 930 nm. The results showed an increase in the transparency of the epidermis by 44 ± 9% within 15 minutes with the use of a solution of HA in combination with sonophoresis before application of PEG-300 to the skin surface. At the same time, when using PEG-300 without preliminary treatment of the skin with the HA, an increase in the transparency of the epidermis was 21 ± 8% within 25 minutes.

Plasmonic sensor of biomarkers based on the Ebbesen effect.

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We present a new method for measuring low concentrations and simultaneously small volumes of fluorescent biomarkers based on the use of the Ebbesen effect of the extraordinary transmission (EOT) of light through an array of nano-holes. In the method the EOT effect is realized at the fluorescence wavelength of the detected molecules with a low transmission of light at the absorption wavelength. The approach allows realizing high level of the sensor sensitivity due to suppression of the inevitable parasitic luminescence of the sensor substrate. The method was demonstrated by detecting an ultra low concentration (at a level of 20 pg/ml (3 p.p.t.)) and an ultra-small volume (about 5 µl) of Cy-5 fluorescent markers in a dimethyl sulfoxide solution.

Application of microencapsulated fluorescent probe SNARF-1 for pH monitoring in circulatory system of fishes and crustaceans *in vivo*

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Methods for the use of polyelectrolyte microcapsules with a biocompatible coating containing a fluorescent dye SNARF-1 have been developed for multiple pH measurements

directly in the circulatory system of fish and amphipods in vivo. The functionality of the techniques for estimating pH changes under stress conditions is demonstrated.

Method of fluorescent imaging-based screening for evaluation of cellular response to drugs by changing cell structures stained with a lipophilic carbocyanine dye DiD

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A method for evaluating cellular responses to chemical compounds by changing the structure ofcells stained with DiD is developed. Phenotypic changes in cell populations were revealed due to nontoxic doses of compounds. The method can be used to test drugs, including chemotherapy drugs.

UV-emitting upconversion nanoparticles for the treatment of estrogen-dependent tumors.

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We have previously obtained ultraviolet-emitting upconversion nanoparticles (UV-UCNP) and applied them for deep cancer treatment with near infrared light (NIR) in vivo. The method is based on active UV- UCNP accumulation in tumor and its emission in UV region upon NIR irradiation. Different endogenous chromophors such as riboflavin or DNA accept UV, leading to cytotoxic reactive oxygen species or pyrimidine dimers formation. As a result we observed tumor regression upon soft and deep-penetrating NIR-irradiation.
Here we demonstrated the enhanced accumulation of riboflavin in estrogen-dependent cell line MCF-7 when supplemented with the hormone. This correlates well with the literature review suggesting that riboflavin binding protein (RfBP) gene expression is under estrogen control. The fluorescence signal of blue-light photoexcited riboflavin has been also 5 times higher in hormone-supplemented MCF-7 comparing to fibroblasts which was detected by flow cytometry. We assume it to be due to enhanced glycolysis of estrogen-dependent cells and propose to take advantage of high enough riboflavin accumulation by estrogen-dependent tumors and to apply UV -emitting upconversion nanoparticles, which match excitation of riboflavin when irradiated with NIR light for tumor therapy.

The study of immunogenicity of microencapsulated fluorescent probes in the primary hemocyte culture of amphipods

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Microencapsulated fluorescent sensors (MFS) are a promising tool for recording the physiological parameters of hydrobionts in dynamics and in vivo. In this work, the primary culture of immune cells of amphipod hemolymph has been obtained for the first time and a low level of immunogenicity of MPS for these animals is shown in this cultu

Possibilities of fluorescent dyes for evaluation of autophagy in HeLa tumor cells under the action of Gratīola officinālis extract.

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Today, the issue of studying the role of autophagy in the development of tumor cell resistance to treatment is topical.

The aim of the study was to study the possibilities of fluorescent dyes for the evaluation of autophagy in HeLa tumor cells under the action of the extract of Gratīola officinālis.

We found that double staining with fluorescent dyes allows effective detection of autophagy in tumor cells.

Hyperspectral imaging microscopy for identification of microorganisms

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Conventional culturing methods for microorganisms detection and species differentiation is known to be time consuming, thus the aim of this work was to use hyperspectral system imaging with CytoViva® dark field condenser as a rapid tool for detection of microorganisms. Hyperspectral imaging is one of the methods that is developed and researched to solve the problems of real-time identification of microorganisms, and provides simultaneously obtain large amounts of spatial and spectral information on the objects being studied. Using the hyperspectral microscopy it is possible to identify the spectral unique profile for studied organism. As model organisms were used microorganisms such as the bacteria Escherichia coli, green microalgae Chlorella vulgaris and yeast Rhodotorula sp. Hyperspectral system imaging the collection of all vis–near-infrared (vis-NIR) spectral data within each pixel of the scanned area and forming a library database. For Chlorella vulgaris was found a bimodal peak at 450 nm and 700 nm of wavelengths. The spectral profile of the Rhodotorula sp. showed a band with a maximum located at 425 nm. The spectrum of Escherichia coli bacterial presented a wide band across the vis-NIR range. The spectral profile of the different cells differs substantially, allowing optical and spectral differentiation. The work was performed according to the Russian Government Program of Competitive Growth of Kazan Federal University and by President of Russian Federation grant MD-6655.2018.4.

Effects of prenatal and perinatal exposure to gold nanoparticles on the internal organs of rat offspring.

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The effects of peroral administration 15 nm and 50 nm gold nanospheres in pregnant and breastfeeding rats were studied on rat offspring. The morphological examination of internal organs of 4-month rats was performed with morphometric analysis. The signs of immune activation in spleen and necrobiotic changes of hepatocytes were noted in rat offspring.

The role of NO in photodynamic therapy.

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NF-кВ участвует в ФД-индуцированной генерации NO. Гуанилатциклаза модулирует генерацию NO. Кальциевые каналы плазматической мембраны играют важную роль в активации nNOS в нейронах и глие речного рака при ФД воздействии. SERCA регулирует уровень ФД-индуцированного NO через активацию nNOS.

Changes in the cell cycle related to Cornelia de Lange syndrome

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The effect of mutation in the regulator of cohesin NIPBL on the organization of sister chromatids in interphase by replicative labeling and SIM microscopy was studied. The change in the distribution of replication patterns in mutant cells in the G2 phase suggests the importance of cohesin in the formation of structural domains of chromatin

Identification of tumor-associated microglia/macrophages by fluorescent microscopy

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Malignant brain tumors and especially gliomas are considered to be the most devastating and dangerous neoplasms. Significant evidence in the literature suggests that Tumor Associated macrophages/microglias (TAMs) play prominent role in cancer proliferation, survival and migration [1]. Therefore, it is highly relevant in terms of cancer treatment to distinguish TAMs from other cells (including tumor cells), detect and subsequently therapeutically affect them. In this study, we report a novel approach of TAMs phenotype identification by means of fluorescent and time resolved microscopy using photosensitizer 5-Aminolevulinic acid-induced protoporphyrin IX (5-ALA induced PpIX).

Experiments were conducted on mature Wistar rats' models with induced glioma C6 brain tumor of high malignancy rate. 5-ALA photosensitive compound stimulates synthesis of photoactive PpIX, which, in turn, is characterized by high fluorescent contrast and enhanced

accumulation in tumor cells and immunocompetent cells involved in metabolic processes of pathologically altered tissue. Photosensitizer was administrated intravenously at 100 mg/kg concentration. Three hours after the injection that corresponds to maximum accumulation point of 5-ALA-induced PpIX in the neoplasm, animals were anesthetized with 60 mg/kg Zoletil (VIRBAC, France). Brain tissue cryosections of 50 µm thickness were obtained using Cryostat Cryo-Star HM 560. For subsequent investigation, samples were placed between superthin cover glasses. The examination of the tumor boundaries against the background of the neighboring normal tissue was performed using laser scanning confocal microscopy (LSM-710-NLO Carl Zeiss, Germany) and Fluorescence lifetime imaging (HPM-100-07Becker&Hickl GmbH, Germany) techniques.

Boarder of tumor and adjoining normal tissue acted like the major zone of interest. PPIX fluorescence was observed within the tumor perimeter, however the most intense signal up to 10 times higher was registered in cells located along the tumor boarder and in some separate single dots within the tumor region. It was concluded that these cells presumably correspond to TAMs whose signature is readily separated from the intrinsic fluorescence signal of tumor and its microenvironment. Fluorescence lifetime distribution of the sample was obtained, lifetime values for TAMs in different locations amounted 0.53÷0.73 ns. In prospect, such an approach will provide a unique ability to discern specific cell types by means of quantitative analysis. The results of the study are relevant both from the fundamental and applied point of view.

[1] D. Hambardzumyan,D.H. Gutmann, and H. Kettenmann. The role of microglia and macrophages in glioma maintenance and progression. Nat Neurosci. 2016 January ; 19(1): 20–27. doi:10.1038/nn.4185

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Study of ATP and chlorine-ion concentration changes in the cytosol of individual cultured neurons during glutamate-induced deregulation of calcium homeostasis.

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Using genetically coded fluorescent protein sensors and synthetic fluorescent Ca2+ indicators it was shown that excitotoxic glutamate concentrations induced in the cytosol of cultured rat cortical and hippocampal neurons rapid acidification, increase in [Cl-], decrease in [ATP] and two-phase increase in [Ca2+]i. Supported by RFBR grants 17-00-00106, 16-04-00792.

Identification of nanoparticles in the organism of nematodes *Caenorhabditis elegans* by using dark-field hyperspectral microscopy

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Every year new nanomaterials are used, the detection of which in living organisms is very complicated. The visualization of particles with a light microscope is virtually impossible, and electron microscopy methods requires multi-stage sample preparation and expensive reagents. High-contrast enhanced dark-field (EDF) microscopy CytoViva allows quickly, easily and efficiently to observe of the nanoparticles within living organisms without using chemical fixation or fluorescent dyes. Spectral curves from libraries can be used to classify or automatically identify particles on a hyperspectral image.

The purpose of our study was to demonstrate the use of EDF microscopy for detection of localisation and aggregation of the nanoparticles inside *Caenorhabditis elegans* microworms. The nanoparticles of natural origin (halloysite, bentonite, montmorillonite, kaolin) or artificial (silver, gold, magnetite) were used as a model nanomaterials. The nanocoated microorganisms (bacteria, algae, yeast) were supplied to the *C. elegans* nematodes as the sole food source.Such microorganisms-carriers of nanoparticles will help to penetrate the particles into the body of nematodes. The cell wall of microorganisms has a different chemical composition and possesses a certain surface charge. The effectiveimmobilization of nanoparticles on microbial cells was carried out by layer-by layer deposition of polyelectrolytes and nanoparticles. Using a dark-field hyperspectral microscopy, we visualized nanoparticles in the whole of digestive system of nematodes. The assessment of the distribution and aggregation of nanoparticles using enhanced dark-field microscopy can be utilized in nanotoxicity studies. A spectral database of various particles has been developed. A method for identifying of nanomaterials in living systems was also developed using the spectral characteristics of nanoparticles. The technique of enhanced dark-field microscopy described in this abstractcan undoubtedly be used to visualize a wide range of nanoparticles in thereal time.

This work was performed according to the Russian Government Program of Competitive Growth of Kazan Federal University and by Russian Science Foundation grant №17-04-02182.

Relevance of the Method for Spheroids Obtaining: Impact on Biochemical and Pharmacological Properties of Tumor Spheroid Models Visualized by FLIM

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The models of spheroids are similar to primary tumors that do not have capillaries, so it is convenient for anticancer drug testing. For the formation of spheroids, various approaches are used, such as the formation in a hanging drop or in rotating vessels. It is the difference in the nature of these spheroids that can largely determine their properties. Multicellular spheroids form in the drop the very next day and are used to study various processes. Rotating spheroids more likely imitate the circulating tumor cell clusters which are considered the oligoclonal precursors of tumors.

We demonstrated the impact of methods for tumor spheroids obtaining on their biochemical and pharmacological properties. We used two approaches of tumor spheroids obtaining, one is suspended drop (multicellular spheroids-MCS) and another is rotation (RCS) in a vessel. Our

results showed that spontaneous caspase-3 apoptosis activation aroused during long term cultivation in RCS and not in MCS by fluorescence lifetime imaging (FLIM) of caspase-3 sensor readouts. The differences of Doxorubicin distribution and anticancer drug responses in two types of spheroids were demonstrated as well by the same method.

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