2012 Workshop on Super-resolution and Life Sciences

3-6 October, Brasov, Romania

PROGRAM
AND
BOOK OF ABSTRACTS

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2012 Workshop on Super-resolution and Life Sciences

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CONFERENCE SCHEDULE

3rd October	
16.00 – 18.00	Registration
18.00 – 21.00	Welcome reception
4th October	
9.00 – 9.30	Opening
9.30 – 11.00	Plenary talks Chair: G. Saavedra **Alberto Diaspro** *Optical Nanoscopy and Super resolution microscopy: variations on the theme* **Colin J. R. Sheppard** *Localization of high aperture beams* **M. Martinez-Corral** *Microscopic and macroscopic imaging throug lightfield technology*
11.00 – 11.30	Coffee break
11.30 – 12.30	Plenary talks Chair: A. Diaspro **Genaro Saavedra** *New architectures in structured illumination microscopy with incoerent light* **Paolo Bianchini** *Fast scanning single wavelength two-photon excitation stimulated emission depletion (SW 2PE-STED) nanoscopy*
12.30 – 14.00	Lunch
14.00 – 16.00	LANIR meeting (for participants in LANIR project only)
16.00	Trip to Poiana Brasov
19.00	Dinner
5th October	
9.00 – 11.00	Plenary talks Chair: C. Sheppard **Thorsten Wohland** *Imaging Fluorescence Correlation Spectroscopy in Live Specimen* **Veronica Lazar** *Microbial biofilms studied by high-resolution imaging techniques*

	Mihaela Gheorghiu *Appraisal of living cells using a high resolution analytic platform* **Radu Hristu** *Optical and morphological characterization of electron beam created surface potential microdomains on hydroxyapatite coatings*
11.00 – 11.30	Coffee break
11.30 – 12.30	Plenary talks Chair: T. Wohland **Stefan G. Stanciu** *Towards semi-automated diagnostic of liver fibrosis by two photon excitation and bag-of-(SIFT)-features* **Brent M. Witgen** *Histological Quantification of Beta Cell Mass using Image Analysis*
12.30 – 14.00	Lunch
14.00 – 15.30	Plenary talks Chair: G. Stanciu **S. den Hoedt** Simultaneous Scanning Electron and Fluorescence Microscopy **Denis E. Tranca** *Scattering near-field optical microscopy for gold nano-particles investigations* **Antonella Chesca** *The Applicability of Transmission Electron Microscopy in Medicine*
15.30 – 16.00	Coffee break
16.00 – 17.30	Round Table: Perspectives of collaboration in the frame of the European Programs
17.30 – 19.30	Historical City tour of Brasov
19.30	Dinner
6th October	
9.00 – 10.00	Plenary talks Chair: M. Martinez-Corral **J. Korczyński** *Microscopy techniques as a tool in signal transduction studies* **George Stanciu** *Real Time Label Free Nanoscopy Using Infra Red Absorption (LANIR)*
10.00	Closing

Optical Nanoscopy and Super resolution microscopy: variations on the theme

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Several methodologies have been developed over the past several years for optical nanoscopy and super-resolution fluorescence microscopy. Among them stimulated emission depletion microscopy (STED), photoactivated localization microscopy (PALM), fluorescence photo activation localization microscopy (FPALM), and stochastic optical reconstruction microscopy (STORM). They have shown great promise for biological and medical research even if having some individual strengths and weaknesses. At the IIT headquarter in Genoa, we have recently developed original approaches aiming to 3D imaging of thick samples (F.Cella Zanacchi et al., Nature Methods (2011); P. Bianchini et al. PNAS, (2012)) and to achieve an important flexibility (Galiani et al., Optics Express (2012). As well, we applied super resolution methods to direct laser writing on nanocomposite materials (B. Harke et al., ChemPhysChem (2012) and to atomic and force spectroscopy (B.Harke et al, Optical Nanoscopy (2012)). This lecture will outline some variations on the theme in optical nanosscopy and super resolution microscopy. Since both fields are comparatively new, scientific discussion would help in focusing on new outstanding goals.

Localization of high aperture beams

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A variety of different measures for the localization of high-aperture scalar wavefields, based on the moments of the pupil, are compared. These are applied to particular examples such as Gaussian beams in the complex focus model, and aplanatic and Herschel focusing systems. These measures can be used to investigate focusing properties of different optical systems, and the use of apodizers and super-resolving masks. The optimum apodizer for achieving minimum axial second moment width is investigated [1].

High numerical aperture optical fibres, planar waveguides or laser diodes with a thin active layer can produce highly divergent beams of radiation. The measurement of values of the beam propagation factor M^2 that are less than unity is discussed, and a method for characterization of the beams proposed.

Reference

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Microscopic and macroscopic imaging throug lightfield technology

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Lightfield technology is a rising 3D imaging technique that can be considered the incoherent version of holography. In this technique the multiperspective information of 3D scenes is stored in a 2D picture. Such picture, composed by a set of elemental images, is obtained through a 2D array of microlenses. The elemental-images set can be used for many purposes. One is the display of 3D color scenes to audiences or much more than one person. Other is the 3D display, with full parallax, in personal monitors, like the screen of a Smartphone, a tablet, or the monitor used by a surgeon in an endoscopic operation.

Other important types of applications are connected with the topographic reconstruction, slice by slice, of the 3D scene. This is especially important in the case of microscopy applications. In this talk, we review the important capacities of lightfield technology and the results obtained by our group, the 3D Imaging & Display Lab.

Simultaneous Scanning Electron and Fluorescence Microscopy

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We have built a platform for combined and simultaneous scanning electron and optical microscopy (SECOM). The platform is built such that different brands of scanning electron microscopes (SEMs) can be easily retrofitted with a wide field fluorescence microscope. The use of vacuum compatible immersion oils makes high NA (up to 1.4) lenses possible. We believe this platform makes correlative microscopy easier and faster, bringing correlative microscopy within the reach of a broad group of researchers.

This talk will touch upon the platform's design and its applications. Preliminary results achieved with the platform will be shown. An outlook will be given on ongoing developments, including the use of fluidic capsules and novel sample preparation methods.

Keywords: integrated correlative microscopy, electron microscopy, fluorescence microscopy, sample preparation

New architectures in structured illumination microscopy with incoerent light

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Structured illumination (SI) techniques provide conventional widefield microscopes with the capability of imaging 2D optical sections from 3D specimens by projecting a stripe pattern onto the sample. A stack of 2D images is recorded after scanning the object axially. By use of a phase-shifting technique, an extended spectrum of the object can be recovered over the image. This completion not only extends the lateral spatial resolution but also provides the system with optical sectioning capabilities [1].

Two different schemes have been proposed for SI microscopy for which the coherence of the illumination field is a critical issue. In the coherent setup a pattern is generated after interference of two or three tilted plane waves. In the simplest two-wave case an axially extended periodic pattern is projected onto the sample [2]. The main drawback of this proposal is that different goals in microscopy, as optimum optical sectioning or maximum lateral resolution cannot be achieved simultaneously, and require different values of the spatial frequency of the illumination. Thus, some tunability of this *carrier* frequency is desirable, which is not easily achievable in the conventional setups. On the other hand, the three-wave interference technique was introduced to reach both goals simultaneously [3], but the presence of the coherent noise in the illumination field can affect dramatically the performance of the technique.

The incoherent SI microscopy proposals generate an axially confined SI pattern by imaging an incoherently illuminated grating onto the 3D sample [4]. This illumination can be used to improve both the axial and transverse resolutions of a conventional microscope. However, for achieving the optimum 3D superesolution it is necessary to use a high spatial frequency grating which image can be strongly degraded by the projection optical system. This decrease in the contrast of the illumination pattern compromises again the signal-to-noise ratio of the reconstructed 3D images.

A new set of techniques is proposed here to try to overcome these limitations. They are based on the use of a Fresnel biprism for the generation of the SI patterns. First, the *coherent* proposal is based on illuminating the biprism by a point source and collimating the resulting interference field [4]. By axially shifting the biprism one can generate the same interference pattern at every transverse plane with the same but tunable spatial frequency. Second, the substitution of the point source for an incoherently illuminated slit allows us to replicate our coherent results but in an *incoherent* way, thus getting rid of the coherent noise. Finally, an extension of this proposal permits to generate an axially limited SI distribution by adapting the flexible scheme in [4] for use with a spatially incoherent source. By a proper design of the source, it is possible to obtain an interference pattern with maximum visibility only at a discrete set of transverse planes. In addition, the spatial-frequency on these *resonant* planes can be tuned by simply shifting the biprism along the optical axis.

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[5] E. Sanchez-Ortiga et al., *Proc. SPIE* **7690**, 769005-1–769005-7 (2010).

Fast scanning single wavelength two-photon excitation stimulated emission depletion (SW 2PE-STED) nanoscopy

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Two-photon excitation (2PE) fluorescence microscopy is a widely used technique and is particularly suitable for three-dimensional (3D), deep tissue and in vivo imaging applications. Unfortunately, although the quadratic dependence of the excitation allows intrinsical rejection of the out-of-focus plane and reduces the width of the focal spot, 2PE cannot be considered a superresolution technique. In fact the "doubling" in wavelength causes that the size of the diffraction spot of excitation light is twice than the one achievable by single-photon excitation (1PE). Recently, 2PE microscopy has been proposed coupled with stimulated emission depletion (STED) technique, combining the advantages of 2PE with the super-resolution ability of STED[1,2]. In this work we present a new class of two-photon excitation - stimulated emission depletion microscope (2PE-STED) exploiting the very same wavelength for excitation and depletion[3]. In general CW-STED[4] is preferred for 2PE imaging due to a simplification of the experimental set-up at the expenses of an increased cost. Unfortunately, the use of two distinct wavelengths for excitation and depletion requires mostly a special optical filter design to make the setup invariable in terms of the choice of the marker dye and potential light beam distortions have to be treated separately. Working with only just one wavelength for excitation and STED one would directly simplify the imaging formation scheme. We propose an imaging method to perform 2PE-STED imaging using a single wavelength (SW) and, consequently, the very same laser source for 2P excitation and depletion. We show that this method allows super-resolved imaging on biological specimens employing a very common fluorophore like ATTO647n achieving a resolution 5-6 times better than conventional 2PE microscopy. Therefore it allows an easy coupling to a conventional commercial confocal microscope. The SW 2PE-STED microscopy is a promising technique to better actively control distortions when imaging thick highly scattering specimens while using the very same wavelength for excitation and depletion.

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Imaging Fluorescence Correlation Spectroscopy in Live Specimen

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Fluorescence Correlation Spectroscopy (FCS) is a powerful tool to study molecular processes over a wide range of times (nanoseconds-seconds) with single molecule sensitivity. However, FCS is performed mostly in a confocal mode preventing large scale multiplexing. By illuminating a thin layer within a sample by either total internal reflection (TIR) or single plane illumination microscopy (SPIM) and using fast and sensitive EMCCD or sCMOS cameras as detectors we can record thousands of FCS curves simultaneously with diffraction limited spatial resolution and a temporal resolution down to 38 □s. This is sufficient to study the movement of proteins within living cells and organisms and provides unique advantages compared to confocal schemes. First, a whole cell cross section can be measured simultaneously giving access to all parts of a cell at the same time; second, the large number of measurements provides excellent statistics; third imaging FCS provides spatial and temporal resolution and thus gives access to spatial organization and dynamics; and fourth, the illumination schemes inflict much less photodamage on the sample compared to confocal techniques, thus providing more measurements per sample.
Here we demonstrate how a SPIM-FCS system is calibrated and how absolute diffusion coefficients can be measured with the system. We will give examples of SPIM-FCS measurements of transcription factors within the nucleus of cells and fluorescent proteins within zebrafish embryos to demonstrate the feasibility of the measurements of fluorescent proteins in live specimen by SPIM-FCS
Imaging FCS with its high time resolution is complementary to super-resolution microscopy and in the future important advantages can be gained by combining the high spatial and temporal resolution of the two approaches.

Microbial biofilms studied by high-resolution imaging techniques

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Microorganisms and microscopes – an indissoluble couple, since 1674 when the Dutch Antonie von Leewenhoeck observed for the first time by his own handcrafted microscope - a magnifying glass, some bacteria harvested from his dental plaque - the most common biofilm. Since then until today a huge progress in microscopy techniques allowed a tremendous development of Microbiology; at present time each microscopic method (optical microscopy – direct and inversed, SEM, TEM, CLSM) is used giving specific information, at different level of organization, from single cells, to adherent social arrangements or biofilms formed on different surfaces. A biofilm is a sessile microbial community composed of cells embedded in a matrix attached to a substratum or interface, the encased cells presenting a modified phenotype, especially with regard to growth rate and gene transcription. A mature biofilm reveals a *citadels*–like association, with *mushroom*, *column* and *pillar*-like structures with spatiotemporal relationships revealed by CLSM, and a synergistic, metabolic cooperation. Biofilms may form on living or non-living surfaces and represent a prevalent mode of microbial life in natural environment, but also in artificial settings. The most important discovery in the last years was that the adherent bacteria are phenotypically deeply different of their counterpart – planktonic or free bacteria. The biofilm formation is considered a survival strategy as the structure is less susceptible to adverse or stress conditions, including all kind of antimicrobials. The high-resolution imaging techniques are also very useful for the study of the influence of different conditions on attachment/ dettachment of biofilms.

Appraisal of living cells using a high resolution analytic platform

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Noninvasive, long term appraisal of cell dynamics holds the promise for development of sensitive and effective cellular platforms suitable to assess the interactions between noxious compounds (including unknown agents) and living cells [1,2] to be implemented in medical and environmental applications.

Further to relatively common combination of impedance and optics (light microscopy) as implemented in ECIS chips, within the International Centre of Biodynamics, we advance novel measurement configurations combined with modeling tools [3,4] for electro-optic evaluation of model cell cultures involving impedimetric, AFM, imaging or Surface Plasmon Resonance (SPR) assays.

Having beta amyloid fibrils – cell membrane interactions as a case study, we reveal cell dynamics in relation to cell attachment, growth conditions [5] and pore formation [4,5,6].

Acknowledgment
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Optical and morphological characterization of electron beam created surface potential microdomains on hydroxyapatite coatings

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Surface properties of hydroxyapatite (HA) such as wettability, chemical composition, electric charges, surface roughness and others, play an important role in many medical and biological applications, especially when using HA in reconstructive orthopedic and dental surgery. The electrical properties of HA have become of interest because it is now well known that local electrostatic charge distribution at the surface can influence the interactions between HA and cells or tissues.

HA thin films have been deposited on Si substrates by spin-coating through a sol-gel synthesis method. The HA surface was irradiated by a focused electron beam and matrices consisting in 2 x 2 points were created.

To investigate the charged areas on the HA surface we have used laser scanning microscopy to collect luminescence images and to analyze the luminescence spectra and atomic force microscopy in order to evaluate the influence of the electron beam on the topography of the sample. An extension of the atomic force microscope, an apertureless scanning near-field optical microscope was used to image surface charge with sub-wavelength resolution.

Histological Quantification of Beta Cell Mass using Image Analysis

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Alterations in beta cell mass occur as part of growth or diseases like diabetes mellitus. The histological assessment of these changes is an important outcome measure in evaluating pathological states as well as therapeutic efficacy. Histological methods for obtaining quantifiable data on these, and other pancreatic markers, are often time consuming and labor intensive. Here we present a method for quantifying the beta cell mass of the rat pancreas using a combination of systematic tissue sampling, immunohistochemistry, automated slide-scanning technology and image analysis. In obese adult male Sprague Dawley rats, beta cells consist of 1.5% of the total pancreas, with an estimate of 24 ± 4 mg (mean ± stdev). Additionally, alpha cell mass was similarly assessed, as 0.25% of the total pancreas, with an estimate of 4.5 ± 1.5 mg. Optimization of these methods have been conducted, and reliability of the mass estimates are enhanced with increased computer automated sampling density. Similar tissue sampling approaches may also be applied to obtain insulin content information. Routine processing of the tissue (cutting and staining) remain hands-on, yet digital slide scanning and image analysis are solely limited to computer processing. Taken together, this histological strategy for obtaining beta cell mass is an efficient method for assessing therapeutic interventions and providing pathological details in a quantitative manner.

Towards semi-automated diagnostic of liver fibrosis by two photon excitation and bag-of-(SIFT)-features

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Liver fibrosis is the excessive accumulation of extracellular matrix proteins including collagen that occurs in most types of chronic liver diseases. Advanced liver fibrosis results in cirrhosis, liver failure, and often requires liver transplantation. Therefore, accurate staging of liver fibrosis is of paramount importance to determine the state of disease progression, responses to therapy, and optimization of treatment to direct disease management. Monitoring liver fibrosis progression by liver biopsy is important for certain treatment decisions, but repeated biopsy is invasive. Laser Scanning Microscopy, in particular with its variants Second Harmonic Generation (SHG) and Two-Photon Excitation (TPEF), represents a powerful tool that can be employed for a non-invasive accurate diagnosis of liver fibrosis. The images collected by SHG and TPEF, can be used in association with advanced image processing/computer vision algorithms for establishing a robust scoring system for diagnosing liver fibrosis that avoids the risk of inter-observer variations and speeds up the diagnostic. The SHG and TPEF data can be regarded as complementary and the difficulties that can occur when using the data collected by one of the two techniques can be compensated by using the data collected by the other. While several robust image analysis methods based on SHG data for the quantification of liver fibrosis progression have already been reported by the group of Prof. Hanry Yu, TPEF data has not yet been exploited at its full potential. Our experiment combines a recent computer vision method, bag of features, and TPEF data, demonstrating the potential of such an approach for the automatic classification of liver fibrosis. Within a Bag-of-Features method an image is represented as an orderless collection of local features. The image features can be regarded as representations of local areas of the image. Further on a dictionary is built by constructing a visual vocabulary which is done by clustering features extracted from a set of training images. Clustering is required so that a discrete vocabulary can be generated from hundreds of thousands (or millions) of local features sampled from the training data. Each feature cluster is a visual word (an element in the visual vocabulary). Given a novel image, features are detected and assigned to their nearest matching terms (cluster centers) from the visual vocabulary. The term vector is then simply the normalized histogram of the quantized features detected in the image. The method that we have experimented revolves around the representation of a TPEF image as a term vector constructed based on the Scale Invariant Feature Transform (SIFT). In the first stage we detect SIFT features from fixed locations in a collection of TPEF images representing four of the five liver fibrosis stages (rat). In the second step the vocabulary clusters are calculated. In the third step we represent a collection of training images (previously classified by histopathologyst) as term vectors based on the vocabulary created at the previous step. An image to be tested against the training data is first represented as a term vector, and then a k-nearest neighbour classifier is employed to find the closest representations in the training set which are further on used by a decision criterion for the classification of the test image. The method yields promising results.

Simultaneous Scanning Electron and Fluorescence Microscopy

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We have built a platform for combined and simultaneous scanning electron and optical microscopy (SECOM). The platform is built such that different brands of scanning electron microscopes (SEMs) can be easily retrofitted with a wide field fluorescence microscope. The use of vacuum compatible immersion oils makes high NA (up to 1.4) lenses possible. We believe this platform makes correlative microscopy easier and faster, bringing correlative microscopy within the reach of a broad group of researchers.

This talk will touch upon the platform's design and its applications. Preliminary results achieved with the platform will be shown. An outlook will be given on ongoing developments, including the use of fluidic capsules and novel sample preparation methods.

Keywords: integrated correlative microscopy, electron microscopy, fluorescence microscopy, sample preparation

Scattering near-field optical microscopy for gold nano-particles investigations

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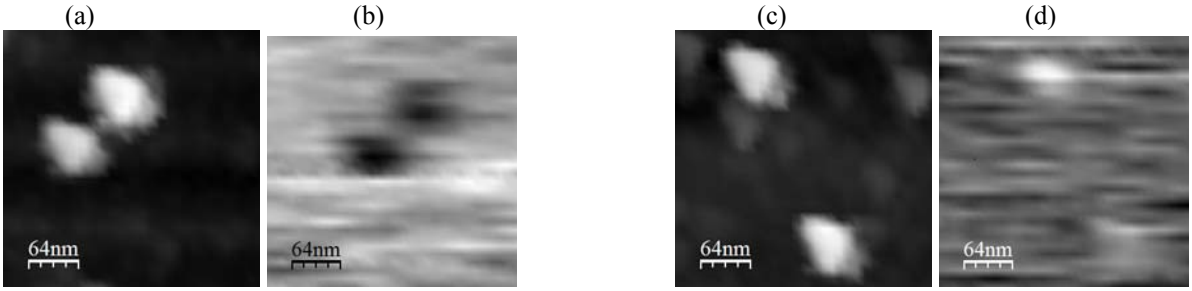
Scanning near-field optical microscopy (SNOM) is a complex technique that offers the possibility for optical sub-wavelength resolution investigations. The most important characteristic of this kind of microscopy is that it can access the evanescent field of a structure under investigation and give at the output a signal which is dependent on the evanescent field.
There are two basic types of SNOM: one which uses a small aperture probe that is held in a close proximity to the sample's surface. The resolution of such a system is limited to about λ/10 because of the needed compromise between laser beam power and the aperture size [1] – reducing the aperture size (for an increased resolution) the laser beam power needs to be increased, but a high light power may damage the probe. The second type of SNOM (scattering-SNOM) uses a metallic AFM probe which is brought in close proximity to a sample surface while a laser beam is focused on the probe's tip. The probe is vibrating next to the sample for harmonic demodulation of the scattered signal. The imaging mechanism in s-SNOM is based on the optical interaction between the tip and the sample, mediated by the evanescent fields. This interaction modifies the amplitude and the phase of the light scattered by the probe's tip and the detection is realized using far-field methods. Our system uses a modified Michelson interferometer to implement a pseudo-heterodyne detection in which the detection signal is given by the interference between the scattered light and a reference beam (at the same wavelength) which is reflecting from a vibrating mirror [2]. This vibration of the mirror modulates the phase of the reference beam.
It was proved – both theoretically and experimentally [3, 4, 5] – that the metallic samples give a high near-field scattered light. In this work we have investigated samples containing gold nano-particles. The image quality and contrast appear to be very sensitive to different values of vibration amplitude and frequency, for both the cantilever and the mirror. In particular, the vibration amplitude of the mirror has a high influence upon the image contrast, because the interference signal depends on the phase difference between the scattered light and the beam reflecting from the mirror.



Gold nano-particles on glass: (a), (c) – AFM topography; (b) s-SNOM, mirror amplitude of 730 nm; (d) s-SNOM, mirror amplitude of 710 nm.

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The Applicability of Transmission Electron Microscopy in Medicine

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Introduction: The aim of the study is to notice the ultrastructural components of the non pregnant uterus and pregnant uterus at due time, using transmission electron microscopy technique. The most important aspect is to observe changes concerning the myometrium, the middle layer of the uterus, in non pregnancy and pregnancy. From this point of view, transmission electron microscopy technique helps in observing the ultrastructural characteristics of the smooth muscle tissue, connective tissue and of the vascular structures, which are different in pregnancy, when appears hyperthrophy and hyperplasia of the myocites and surrending components.

Material and Method: The investigations were made on human fresh material taken by the specialty services of obstetrics-gynecology, with the contribution of the pathology service within Clinic Hospital of Obstetric-Ginecology "Dr. I. A. Sbârcea" Brasov and of the specialized study laboratories of the Cell and Molecular Medicine Department within "Carol Davila" Medical University of Bucharest and Faculty of Medicine from Brasov.

The study was made according to the Medical Ethics principles. The tissue samples of myometrium were prepared for transmission electron microscopy technique. They were fixed in 4% glutaraldehyde in cacodylate buffer, incubated in Gomori environment and postfixed in 1% OsO4, dehydrated with ethanol and embedded in epon. The sections were examined by an electron microscope Philips 300. There were used lots of women in non-pregnancy and pregnancy at due time, and non-pregnancy and pregnancy control lot women. The study has been done, for observing the changes in the ultrastructure of the myocites, connective cells and blood vessels connected with the hypertrophy and the hyperplasia of myometrial components in pregnancy.

Results: The ultrastructural images shows the junction relations between the uterine smooth muscle fibers, the neighborhood relations between connective cells as fibroblasts, the most important hormone – sensitive type of connective cells, with particularities in non-pregnancy and pregnancy. With electron microscopy technique was possible to show the intercellular junctions between myocytes, connective tissue and blood vessels.

Conclusion: The transmission electron microscopy technique highlighted ultrastructural aspects of the smooth muscle fibres, of the connective cells and of the vascular structures of the myometrium of the non pregnant and pregnant uterus. During the physiological pregnancy the hypertrophy of the myometrium take place and also can be noticed the hyperplasia of the muscle fibres, as one of the reason for the present study, observed using electron microscopy technique.

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Microscopy techniques as a tool in signal transduction studies

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Various microscopy techniques were applied to evaluate experimentally induced changes in glioma C6 cells actin cytoskeleton and to compare changing status of this cytoskeleton to the morphology, migration, adhesion mode and calcium response. We investigated the crosstalk of signaling pathways activated by $P2Y_2$ receptors and regulating the functional state of actin cytoskeleton, namely: RhoA/ROCK, Rac1/PAK and calcium pathways.

To evaluate molecular mechanism of this crosstalk, phosphorylation level of two actin-binding proteins – cofilin and myosin II was studied. The phosphorylation level of these proteins was estimated by measuring the intensity of immunofluorescence staining in confocal microscopy by ImageJ software. The interaction between $P2Y_2$Rs and $\alpha_V\beta_5$ integrins, needed for activation of G_O protein, was analyzed using FRET technique and colocalization methods in confocal microscopy equipped with LAS AF software. The effects observed on the molecular level affect cell morphology, migration, adhesion, cell surface mechanical properties and calcium response. To control morphological changes of the cells, xyz scan of the specimen was performed by confocal microscopy and 3D cell shape visualization was analyzed. Glioma C6 cells migration was examined by direct tracking method using DIC Nomarski contrast microscopy and MetaMorph software. The IRM (Interference Reflection Microscopy) technique was applied to study area of cell adhesion. Atomic Force Microscopy (AFM) was used to compare cell surface stiffness. Calcium response after stimulation of $P2Y_2$ receptor with UTP was imaging on subcellular level by Fura-2 ratiometry on fluorescence microscopy.

Our results indicate that in glioma C6 cells ROCK inhibition decreases cofilin and myosin II phosphorylation. These changes cause: rounding up the cell, changes in actin cytoskeleton reorganization, decrease the speed of directional migration and increase the local cell surface stiffness. We have found that effect of ROCK inhibition can be compensated by $P2Y_2$R induced signaling pathways: Rac1 related and calcium pathways. Changes in cell morphology depend on cofilin, myosin II and $\alpha_V\beta_5$ integrins activation level [1].

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Real Time Label Free Nanoscopy Using Infra Red Absorption (LANIR)

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IR microscopy is a chemical imaging technique that maps the spatial distribution of certain chemical transition which is identified by a well-established and almost universally used IR spectroscopy technique. This technique traces the chemical fingerprint of a variety of biological and nonbiological materials. It means, no chemical labelling is required for molecular identification (label-free). This is highly advantageous especially in detecting small and subtle chemical changes that can potentially have severe consequences e.g. in chemical change in the amyloid protein structure that causes Alzheimer's disease.

Breaking away from the diffraction limit of infra-red (IR) radiation in far-field microscopy has been considered impossible due to the inherent absorption of IR radiation by materials at intensities required to achieve high resolution. The best resolution offered on commercial table-top IR microscopes is currently over 20 microns only. Even with high intensity synchrotron sources, the best resolution obtained in IR microscopy is 2-4 micron, which is still diffraction limited.

Vibrational spectroscopies such as infra-red (IR) absorption, coherent anti-Stokes Raman, sum-frequency generation, stimulated Raman spectroscopy are today well established and capable of revealing the chemical structure of materials. These techniques offer diffraction limited spatial resolution, and high fidelity images can be generated. Sub-diffraction resolution for these techniques generally involves nanoscale near-field probes, and the approach remains thus suitable for surface characterization but appears unpractical in other circumstances.

Here we present a conceptual framework of Infra Red Nanoscopy (IRN) that breaks away from the diffraction limit by exploiting the reversible saturation of vibrational transitions, and the experimental feasibility of the concept is rationalized. It is argued that IRN has the potential for improving the lateral resolution of IR micro-spectroscopy from the diffraction-limited current state-of-the-art down to the nanoscale. Chemical characterization and structural imaging under ambient conditions with sub-micron and nanoresolution can significantly advance our understanding of biological processes at the sub-cellular level, provide insight into early stages of diseases such as Alzheimer's disease and lung cancer, and contribute to the improvement of therapeutic drugs and to the assessment of the real impact of nanomaterials on health and safety.

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