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**CORRELATIVE MICROSCOPY IN LIFE
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**CORRELATIVE MICROSCOPY IN LIFE
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Oral presentations1

TERMINAL DIFFERENTIATION OF MOUSE BONE MARROW-DERIVED EOSINOPHILS WITH IL-33 INCREASES THEIR SURVIVAL CONTRIBUTING TO THE ANTI-TUMORAL ACTIVITY

C. Afferni^{2*}, V. Lucarini¹, G. Ziccheddu¹, C. Buccione¹, S. Andreone¹, F. Mattei^{1*}, G. Schiavoni^{1*}

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We demonstrated that pulmonary conditioning with IL-33 protected mice from the onset of experimental pulmonary melanoma metastasis through selective recruitment in the lung of eosinophils, via IL-33 specific receptor-mediated signaling.¹ Because tumor-infiltrating eosinophils have been associated with a good prognosis in several clinical solid cancers, including colorectal, oral squamous and prostate carcinomas,² we further investigated the mechanisms by which IL-33 promotes the anti tumoral activity of eosinophils. *In vitro* differentiation of bone marrow-derived eosinophils (BM-EOs), flow cytometry, histochemistry, *in vivo* Winn Assay. Since IL-33 was shown to inhibit eosinophilopoiesis in BM progenitors when given at early times of culture,³ we developed a protocol for eosinophils differentiation by culturing BM cells in presence of IL-5 for the first 10 days of culture followed by IL-33 for the last 6 days of culture. This IL-33-based protocol generated similar numbers of eosinophils as the IL-5-based protocol. Of note, the eosinophils terminally differentiated with IL-33 (IL-33 EO) exhibited a much more activated phenotype with respect to eosinophils differentiated with IL-5 for the whole culturing time (IL-5 EO), as shown by higher side scatter and by up-regulation of CD69 and CD11b expression. Furthermore, IL-33 EO exhibited significantly higher cytotoxic activity, with respect to IL-5 EOs, against target B16 melanoma cells, as revealed by flow cytometry-based apoptosis assay, which correlated with increased survival of co-cultured IL-33 EOs. Notably, this effect occurred through an adhesion-dependent mechanism involving up-regulation of ICAM-1 and CD18 by IL-33. Consistently, co-injection of IL-33 EO, but not IL-5 EO, with B16 melanoma cells into syngeneic mice substantially delayed tumor uptake, without perturbing the tumor immune environment, suggesting a direct anti-tumor activity of eosinophils following IL-33 activation. The increased survival rates of IL-33 EO may further support the efficient killing of target melanoma cells. Our results underscore that terminal differentiation of BM-EO with IL-33 results in the generation of activated eosinophils endowed with potent anti-tumoral cytotoxic activity and closely resembling *in vivo* expanded eosinophils following pulmonary conditioning with IL-33.

The authors wish to thank Dr. Mario Falchi for technical support in microphotography.

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INVESTIGATING CANCER CELL BEHAVIOR USING CORRELATIVE IMAGING BY HOLOGRAPHIC MICROSCOPY AND FIB-SEM TOMOGRAPHY

J. Balvan,^{1,2} K. Rosikova², J. Javurek², M. Masarik¹, A. Hampl¹, V. Kostal²

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Multimodal holographic microscopy (MHM) has become a unique method for label-free assessment of cellular dynamics. High contrast, minimal phototoxicity, and extreme sensitivity for the smallest changes in cellular mass are the key advantages over the classical bright field microscopy. On the other hand, the connection between the morphological data recorded by MHM and the cellular ultrastructure is still limited. Therefore, correlation of MHM data with focused ion beam scanning electron microscopy (FIB-SEM) is essential for detailed interpretation of the observed behavior. As the serial FIB-SEM tomography has been routinely used for providing 3D reconstructions of cellular morphology, it makes it an ideal technique for justifying the cellular mass data obtained by MHM. Here, we used a correlative MHM-FIB-SEM workflow for the dynamic, label-free visualization of cancer cell behavior with its subsequent ultrastructural analysis. Using the time lapse MHM microscopy, we were able to identify specific cellular events of interest (e.g. morphological changes or cell-cell interactions) within the cancer cell population. After recording the MHM data, the cells were fixed, resin-embedded and loaded into a FIB-SEM workstation. Subsequently, the regions of interest were identified according to the MHM data and analyzed by high-resolution FIB-SEM 3D tomography. The presented combination of label-free imaging MHM and FIB-SEM analysis offers a deep insight into studying cancer cell heterogeneity. Rare cells with unique proliferative properties can be identified and characterized in large populations of cancer cells. This in turn can lead to better understanding of many cellular mechanisms of tumor cells (e.g. resistance to chemotherapy).

A CASE STUDY OF CORRELATIVE APPROACH TO 3D MICROSCOPY: THE SILICON NANOWIRES

L. Boarino¹, N. De Leo¹, F. Ferrarese Lupi¹, E. Cara¹, M. Fretto¹, M. Dialameh¹, A. Chiodoni², C. Fleischmann³, W. Van der Vorst³

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In recent years a great effort has been devoted to silicon nanowires (SiNWs) research. In particular, it is possible to find many literature reports on their fundamental properties, like electron and thermal transport,^{1,2} but also in energy production in photovoltaic and solid state batteries.^{3,4}

Silicon nanowires have been fabricated by the combined approaches of large area self-assembly nanolithography and Metal Assisted Chemical Etching (MACE). Depending of the initial substrate doping and etching solution composition, the SiNWs can be porous or completely crystalline, this affects the major physical-chemical properties like electrical, thermal transport and reactivity to external environment. A study of these parameters of a single SiNW has been carried out in INRiM in the framework of European projects and collaborations. In both the studies, the local morphology strongly influences the conduction pathways in the

SiNW, in terms of bottlenecks for carrier or phonon transport, so a detailed 3D microscopic analysis of the wires is mandatory. Simple SEM and TEM analysis is not sufficient and both TEM and Atom Probe tomography is necessary to validate the calculation codes used to simulate the system. Preliminary results of these correlated microscopies will be presented in the talk, together with a general view of the correlative imaging techniques under study in the EMPIR project 14IND01 3DMetChemIT and others, in the framework of the European Metrology Programme.

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ULTRASTRUCTURAL ANALYSIS OF CELL SENESCENCE FEATURES IN INDUCED PLURIPOTENT STEM CELLS

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Understanding structural details down to the nanoscale is paramount to any scientific purpose, ranging from materials science to biomedical research. Focused ion beam/scanning electron microscopy (FIB/SEM) is a technology used by research laboratories and companies worldwide. Despite its great potential, applications are limited to particle analysis and material characterization, and only occasionally used for biological studies. Induced pluripotent stem cells (iPSCs) are a promising tool in biomedical research thanks to their self-renewal and differentiation abilities. Human iPSCs constitute an unlimited source of patient-specific cell types used for disease modeling, drug screening and regenerative medicine¹. Though extensively characterized from a molecular/genetic point of view, ultrastructural studies are still few, particularly on the morphological changes in iPSCs, as a function of culturing time. Despite general concept that iPSCs can be indefinitely maintained in culture, mitochondrial alterations have recently been found in long-term cultured iPSCs². To investigate senescence processes, we comparatively analyzed iPSCs kept in culture for 1, 6 and 12 months, focusing on mitochondrial morphology. FIB/SEM micrographs from 1-month-cultured iPSCs show few mitochondria displaying immature-like shape, poorly developed *cristae* and perinuclear localization. Conversely, 6- and 12-month-cultured iPSCs display structurally mature mitochondria, widely distributed in the cytoplasm showing an elongated morphology with numerous distinct *cristae*. Moreover, autophagosomes detected in mid-to-long-term cultured iPSCs, are index of ongoing senescence supporting the previously hypothesized idea that long-term iPSCs maintenance may alter mitochondrial status. Based on the key role of mitochondria in iPSCs pluripotency, differentiation and reprogramming, we suggest that these important properties may be progressively lost during culturing time, with relevant impact on their potential use for cell therapy.

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AN ANALYTICAL JOURNEY FROM 4D LIVE CELL IMAGING TO TRANSMISSION ELECTRON MICROSCOPY. FAST, RELIABLE AND TRUSTWORTHY

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3D Cell Explorer and the TEM are the means used to deliver Correlative analytical measures of one cell, we have been able to compare the 3D Cell Explorer's acquisition with those of the TEM. As the TEM has a much higher resolution, the comparison could help for the identification of organelles shown in live cell imaging in 3D and 4D with 3D Cell Explorers detection. It could also serve as a proof that what is being detected by the 3D Cell Explorer is truly what is present within the cell. For those working with an Electron Microscope, the 3D Cell Explorer could be used to identify a cell or area of interest before committing to a labour intensive imaging session. Also, the 3D Cell Explorer could be a simpler, safer, and much faster alternative to Electron Microscope for those imaging biological samples, depending on the desired resolution. Best option we have had tested is to have the 3D Cell Explorer as main actor in the workflow journey that ends in TEM or SEM higher resolution imaging.

3D HDO-CLEM: CELLULAR COMPARTMENT ANALYSIS BY CORRELATIVE LIGHT-ELECTRON MICROSCOPY ON CRYOSECTIONS

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Fundamental to obtaining a depth-understanding of the function and structure of cells is the ability to study and correlate their molecular topography with the ultrastructural morphology, for example, to visualize the position of a given protein relative to a given cell compartment and its morphology. Standard fluorescence light microscopy (FLM) relies on simple sample preparations, and localizes proteins in living or fixed cells with a resolution in the range of few hundred nanometers, allowing large field of view. However, FLM is unable to visualize the unlabeled cellular context. On the other hand, electron microscopy (EM) techniques reveal protein topology with the resolution in a range of a few tens of nanometer, retains the cellular context, but can only be applied on a limited field of view. Therefore, both approaches present shortcomings, in terms of field of view, statistical output, resolution, sample preparation, and context analysis, that can likely complement each other. To bridge the gap between FLM imaging and EM, several laboratories have developed methods for correlative light-electron microscopy (CLEM). In a nutshell, CLEM enables one to investigate the same exact region of interest utilizing the two microscope platforms, and thereby virtually combine their capabilities. We describe a protocol based on immunolabeling of Tokuyasu cryosections that allows correlation of LM and EM images with excellent preservation of cellular ultrastructure. We will refer to this method as high-data-output CLEM (HDO-CLEM). The major benefits of HDO-CLEM are the possibility to (1) correlate several hundreds of events at the same

time, (2) perform three-dimensional (3D) correlation, (3) immunolabel both endogenous and recombinantly tagged proteins at the same time, and (4) combine the high data analysis capability of FLM with the high precision of transmission EM in a CLEM hybrid morphometric analysis. We have identified and optimized critical steps in sample preparation, defined routines for sample analysis and retracing of regions of interest, developed software for semi/fully automatic 3D FLM reconstruction and set the basis for a hybrid light/EM morphometry approach.

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THE EXTRAORDINARY MICROSCOPE: MULTIMODAL AND CORRELATIVE APPROACHES IN NANOMEDICINE

A. Diaspro

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The sentence “*microscopium nominare libuit*” referred to the Galileo Galilei “occhialino” allowed to cry the name Microscope. Developments were amazing coupled to the unique possibility of studying living systems and with some biological revolutions like the advent of green fluorescent proteins. Phase contrast, confocal and multi photon microscopy were only a prelude for the super resolved approaches. Today, in the era of super resolution, we have a continuous growth of variations on the theme. Optimized exploitation of microscopy data, from lifetime to polarization signatures, and new approaches for extending the knowledge about living systems can be integrated in a new paradigm for the microscope. A multimodal microscope tunable on the scientific question and posing scientific questions about light-matter interactions. As well, correlative microscopy coupling optical, scanning force and electron microscopy methods enhances the capabilities of the modern microscope. For this reason I would like to consider a slight change about the Galilei’s microscope sentence rephrasing it into “*microscopium EXTRAORDINARIUM nominare libuit*” referring to the modern optical microscope we have in our hands.

NEW TOOLS AND PROTOCOLS FOR CORRELATIVE MICROSCOPY APPLICATION TO BIOMEDICAL RESEARCH

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Correlative light electron microscopy (CLEM) combines the advantages of light and electron microscopy, thus making it possible to follow dynamic events in living cells at nanometer resolution. Various CLEM approaches and devices have been developed, each of which has its own advantages and technical challenges. There are two principal types of CLEM: fluorescence/confocal microscopy with transmission electron microscopy (TEM) or scanning electron microscopy (SEM). The first has seen the development of various protocols and devices, and is widely used in biological laboratories but the second is still relatively undeveloped and one of its prevailing limitations is the lack of substrates that are optimally suited for relocating the sample when switching

from one imaging mode to the other. In the attempt to overcome these limitations, in our experimental CLEM set-up, we first have grown cells on laser-patterned aclar film^{1,2} and then we have developed transparent, metal-patterned glass coverslips³ that are ideal for high-resolution confocal microscopy, allow cell growth and proliferation, are resistant to electron microscopy sample preparation procedures, and provide optimal contrast for SEM location. Here describe the generation of our customized patterned glass substrates, which improve the feasibility of correlative fluorescence/confocal and scanning electron microscopy and their use in combination with immunolocalization of membrane antigens at both confocal microscopy and SEM level⁴.

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CORRELATIVE X-RAY MICRO TOMOGRAPHY AND TEM MICROSCOPY ON BIOLOGICAL SAMPLES FOR THE STUDY OF COMPLEX PATHOLOGIES

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Correlative approaches are a powerful tool in the analysis of biological samples, since they take advantages at the same time from the information achievable with different imaging techniques. Here we report the optimization and the application of a novel correlation technique between computed X-ray microtomography (micro-CT) and transmission electron microscopy (TEM) in the study of neurodegenerative diseases. To apply different imaging techniques on the same sample, its preparation has to be optimized accordingly. For the embedding of a TEM biological sample, the best efficiency of a chemical protocol is achieved for tissue blocks of 1 mm³, at most. With larger dimensions, a uniform fixation and contrast is challenging. Starting from an optimal embedding protocol for TEM analysis, we modified the different steps of chemical preparation (protein and lipid fixation, contrast) in order to obtain the best compatible with both the imaging techniques here applied. We developed three tissue and size-dependent protocols: 1) small-size protocol for the mouse sciatic nerve (diameter < 1 mm, length ~ 5 mm); 2) medium-size protocol for the mouse spinal cord (diameter ~ 1.5 mm, length > 1 cm); 3) large-size protocol for the rat spinal cord (diameter ~ 4 mm, length > 1 cm). Once the samples are correctly prepared, it is possible to image them with both the micro-CT and TEM at a state of art level. In the 3D reconstructed maps, it is possible to locate with micrometric resolution the volumes of interest (VOIs) for TEM ultrastructural analysis. Here we show a proof of concept of this application: in the 3D rendering of a sciatic nerve of the Twitcher mouse, a model for the Krabbe disease, it is possible to identify some immune infiltrating cells and selectively cut the sample in thin slices across these VOIs for the ultrastructural characterization with the TEM, avoiding a challenging and time consuming serial sectioning. This correlative technique can be applied in the study of those pathologies in which the hallmarks are localized in few confined regions instead of being spread in the entire organ. We are actually working in the characterization of EAE (Experimental Autoimmune Encephalomyelitis) spinal cord tracts, a rat model of the Multiple Sclerosis.

CORRELATIVE LIGHT AND ELECTRON MICROSCOPY IN BIOLOGY

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In recent years correlative microscopy, combining the power and advantages of light and electron microscopy, has become an important tool for biomedical research. Light microscopy has the advantage that cellular processes can be observed in life imaging. Cell movements and responses to chemical effectors can be monitored by phase contrast imaging or specific proteins can be labelled by genetically expressed fluorescent proteins, such as GFP^{1,2}. Next to life cell imaging, light microscopy has the advantage of easily searching large areas, even volumes, for the cells of interest, e.g., a special cell type in tissue, astrocytes in brain.³ Also on thin sections, the low magnification of light microscopy and therefore ease of searching large areas are very beneficial to speed-up the analysis of rare events⁴. Electron microscopy reveals the cellular ultrastructure at high resolution and individual organelles, even large protein polymers like cytoskeletal filaments or ribosomes can unequivocally identified. Proteins of interest can be labelled with colloidal gold particles and localised within the resolution of the labelling system, 5 - 20 Å to the visible ultrastructural features within the cells. Searching for a few gold particles within a few cells of a large tissue, however, is very cumbersome and can be extremely time consuming. Seen the advantages of light and electron microscopy suggests that the optimal approach is to combine both techniques for cell biology research. Life cell imaging and the localisation of rare cellular events are followed and identified by (fluorescence) light microscopy, the high resolution data and fine localisation to cellular substructures are done by electron microscopy.

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APPLICATIONS FOR 3D CHARACTERIZATION IN THE LIFE SCIENCES. ILLUMINATION CORRELATIVE RESEARCH USING LIGHT, X-RAY, AND ELECTRON MICROSCOPY

L.O. Kautschor

Carl Zeiss Microscopy GmbH

X-ray tomography has emerged as a new powerful imaging technique that obtains 3D structural information from opaque samples under a variety of conditions and environments. It has rapidly become an accepted laboratory technique offering quantitative information in life sciences. We present ways in which non-destructive 3D volumetric information, obtained via laboratory nanoscale and sub-micron X-ray microscopy (XRM) are increasingly used to probe scientific questions as a complement to Electron- and Light-based microscopy methods. These correlative methods, relating to XRM, provide an opportunity to study materials evolution at multiple length scales in 3D and utilize this information to inform or guide *post mortem* analysis to be most efficient. In life sciences, correlative microscopy methods have existed for decades in various forms. One remaining challenge is to

identify practical methods of localizing the same feature in multiple microscopes in 3D. XRM presents an additional opportunity to bridge the length scales between LM and EM and ease the 'needle in a haystack' navigation problem. Recently, XRM techniques acting as a bridge between light- and electron- microscopy have acted as an efficiency multiplier to make 3DEM methods highly efficient and targeted, be pre-defining the buried volumes of interest.

CORRELATIVE IMAGING WORKFLOWS ACROSS SCALES: A POWERFUL APPROACH FOR CELL AND TISSUE STUDIES

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Visualizing the three-dimensional (3D) architecture of cells and tissues is essential for understanding relationships between structure and function in biological systems. Correlative imaging with multiple techniques provides valuable comparative information as well as easy targeting of regions of interest at different scales. In the last decade there has been a significant development in SEM-based methods for 3D reconstruction of large tissue volumes. Serial section techniques, previously the domain of specialists, are becoming increasingly automated due to the development of systems such as serial blockface imaging and focused ion beam scanning electron microscopes. These changes are quickly broadening the scope of life sciences to which volume electron microscopy techniques can be applied. FIB-SEM tomography is a powerful approach for 3D imaging of biological samples, allowing fully automated high-resolution imaging of tissue volumes at subcellular level, down to a bilayer resolution. Imaging of the block face with a scanning electron beam and milling away thin sections of tissue with a precise FIB, allows automated imaging and collection of 3D data to visualize and reconstruct 3D ultrastructural and spatial organizations of intricate networks. Serial Block-Face SEM (SBF-SEM) on the other hand involves combination of *in situ* sectioning and imaging of plastic embedded tissue blocks within the SEM vacuum chamber,¹ allowing for automated imaging and subsequent reconstruction of large tissue volumes. SBF-SEM is a particularly versatile and accessible technique for the collection of 3D EM data in life sciences research covering a broad range of applications such as the study of subcellular features, the analysis of cell-to-cell interactions and the investigation of tissue samples and small model organisms. The VolumeScope, Thermo Fisher Scientific's solution for large volume analysis, embodies the versatility and accessibility of the technique with its tight integration of a hard- and software. The VolumeScope tightly integrates SBF-SEM and Multi-Energy Acquisition for isotropic resolution, while automating and streamlining the complete 3D imaging workflow. The system can analyze large area/volume acquisitions by automatically tiling multiple fields of view into a large composite image. It can also import and overlay images from light microscopes to allow direct targeting of regions of particular interest based on fluorescence staining or other LM techniques. An automated, lightweight, in-chamber microtome can be easily removed within minutes to quickly change to other SEM applications. Here, we will show how it enables straightforward, reliable and isotropic 3D volume acquisition, how its data integrates into analysis workflows using Amira and the platform's benefits beyond SBF-SEM (e.g. array tomography and STEM).

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APPLICATION OF CORRELATIVE MICROSCOPY TO STUDY TUNNELING MEMBRANE NANOTUBES

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Communication between cells is crucial for unicellular organisms, especially when they are forming a multicellular structure, termed biofilm, and for all multicellular organisms.¹ There are different mechanisms of cell-to-cell communication, from the secretion of molecules in the extracellular space where they find their targets by diffusion, to the transport of signalling molecules through communication junctions, such as gap junctions and plasmodesmata.^{2,3} Recently, new mechanisms of cell-to-cell communication were proposed when extracellular vesicles⁴ and tunnelling membrane nanotubes⁵ (TnTs) were discovered. The latter are thin, tubular connections that mediate intercellular exchange of cargoes including organelles, proteins, RNAs, miRNAs, ions, bacteria, viruses, and prions between connected cells.⁶ Since they are very sensitive to mechanical stress, light exposure, and chemical fixation, many basic characteristics of TnTs are still poorly known. In this study, we applied correlative microscopy to control the process of sample preparation and to analyze molecular, functional and ultrastructural characteristics of TnTs. Correlative phase-contrast and fluorescence microscopy showed that less than 30% of TnTs existing in live cells is still preserved after immunolabeling procedure. Furthermore, normal and cancer urinary bladder epithelial cell TnTs distinguish in dimensions as well as in the arrangement of cytoskeletal elements and their representative motor proteins. Correlative light and electron microscopy tomography revealed 3D reconstruction of the TnT attachment to a target cell, though it was time consuming and the procedure still contains challenges needed to be solved in the future. In conclusion, correlative microscopy approach exposed novel TnT characteristics, however for additional detailed spatio-temporal 3D reconstruction of TnTs, further dedication, expertise, time and experiences are needed.

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PREPARATION WORKFLOWS FOR CORRELATIVE MICROSCOPY

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A large amount of dyes and probes are now available to localize molecules in tissues and cells. However, accurate localization of these molecules is limited by the spatial resolution of light microscopy techniques. Although super resolution techniques are closing the gap, they do not allow scientists to observe their markers in a cellular or ultrastructural context. In contrast, electron microscopy is the ideal technique to study cell and macromolecular structures up to the Angstrom level. CLEM combines the advantages of both techniques, allowing scientists to spot cellular structures and processes of interest in whole cell images with LM and then zoom in for a closer look with EM. This dual examination

provides valuable complementary and often unique information. A recent approach is to carry out both EM and FM on the identical sample, once it has been prepared for EM. This procedure provides high correlation accuracy. It can be applied to samples embedded in acrylic resin, where the fluorescence is preserved. This usually requires high-pressure freezing, freeze substitution and polymerization at low temperatures. A similar strategy can also be used on vitrified samples. Leica's unique CRYO-CLEM solution allows to automatically scan the vitrified sample and generate a high resolution overview image. The CLEM viewer allows fast and precise location of points of interest. These CLEM coordinates are transferred to the TEM for correlation. During this lecture an overview will be given of different specimen preparation workflows for CLEM experiments and the Leica Cryo CLEM will be shortly introduced.

CORRELATIVE MICROSCOPY IN BIOMEDICINE: FROM THE SLOW BEGINNING DECADES AGO TO THE RAPIDLY EXPANDING LEADING EDGE OF TODAY

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Correlative microscopy (CLEM) combines video-light microscopy with electron microscopy (EM) to integrate information about the dynamics and the ultrastructure of cellular objects of interest. Evolved from a relatively simple beginning in our laboratory¹ CLEM it is now a sophisticated technology that can address different types of biological problems. The assembly and disassembly of protein complexes plays a central role in all cell functions, hence understanding cells requires visualizing protein complexes with spatio-temporal precision. Current fluorescence microscopy can efficiently determine localization and dynamics of individual proteins, but its ability to visualize complexes is limited to binary interactions and relies on complex manipulations. Here we propose a combination of antibody (Fab)-based probes with microfluidics technology to overcome these limits. The key developments are innovative fluorescent probes that allow detection of protein-protein interactions by both long-range FRET measurements and by EM (being developed), coupled with an automated microfluidics platform for multiple serial observation (multiplexing). Microfluidics-based multiplexing FRET-EM microscopy can be designed to focus on complexes associated with the activation of oncogenic signaling pathways or of gene regulatory networks in cancer and can significantly expand the range and power of diagnostic approaches applicable to cancer biopsies.

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VISUALIZING FLUOROCHROME-LABELLED NANOPARTICLES AND FLUORESCENT FREE MOLECULES AT TRANSMISSION ELECTRON MICROSCOPY BY DIAMINO BENZIDINE PHOTO-OXIDATION

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Thirty-five years ago, Maranto¹ demonstrated that diaminobenzi-

dine (DAB) and light illumination can be used to convert fluorescence into a stable signal suitable for examination in bright-field microscopy. In fact, when a fluorophore is appropriately excited by light, DAB is oxidized into an electron-dense osmiophilic product, which precipitates in close proximity of the fluorophore molecules, thus allowing their precise spatial location. Based on this evidence, DAB photo-oxidation has successively been applied to a wide variety of fluorochromes to correlate light and transmission electron microscopy (TEM). The high sensitivity of photo-oxidation makes it a reliable method to accurately detect *in situ* fluorescent molecules even in low amounts. We used this cytochemical method for innovative applications at TEM i.e., for tracking the intracellular fate of fluorescently labelled nanoparticles, and for visualizing the subcellular distribution of dispersed fluorescent molecules (photo-active molecules suitable for photo-dynamic therapy or cell membrane binding dyes). We were thus able to detect at high resolution fluorescent molecules either embedded into nanomaterials or inside membrane-bounded organelles, or at the surface of the plasma membrane, or even free in the cytosol. In particular, DAB photo-oxidation allowed elucidating the internalization mechanisms, the intracellular distribution and the degradation routes of different nanoparticle types^{2,3}. The ultrastructural detection of photo-active molecules provided direct evidence for the lethal multiorganelle photo-damage occurring after cell irradiation⁴. Finally, by DAB photo-oxidation of membrane-labelling fluorochromes it was possible to follow the intracellular fate of endocytosed membranes and to identify the involved subcellular compartments⁵.

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CORRELATIVE LIGHT-ELECTRON MICROSCOPY IN COMBINATION WITH THREE-DIMENSIONAL ANALYSIS OF BIOLOGICAL SAMPLES

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New techniques for 3D analysis have allowed researchers to get additional information about the three-dimensional organization of various biological structures with high resolution. On the other hand, there are several methods of three-dimensional electron microscopy (3DEM) that have largely overlapping capabilities, requires a careful comparison of these methods, identify their strengths and weaknesses, so that on this basis to make recommendations on areas of their application in morphology, cell and molecular biology. Here, we provide an overview of new methods of 3DEM suitable for analysis of biological ultrastructure, analyses the principles, advantages and disadvantages of these methods, as well as the stated area of application. In particular, we evaluate the combination of 3DEM with correlative light electron microscopy (CLEM). Examples of application of 3DEM in combination with CLEM will be presented. We also formulate the future direction of development of electron microscopy.

CORRELATIVE MICROSCOPY AS A POWERFUL TOOL TO CHARACTERISE STRUCTURAL, COMPOSITIONAL AND FUNCTIONAL PROPERTIES

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In the last decades, correlative light-electron microscopy (CLEM) raised its role in the scientific research community among different fields (biomedical, automotive, aerospace, microelectronics) combining the advantages of live imaging (optical microscope) and the high-resolution information (electron microscope).¹⁻⁴ The interactions between probe and sample has been then further improved exploiting the powerful synergy of different imaging systems, e.g. EDS, XRF, WDS, AFM due to the possibility to couple the obtained information and combining them in a more detailed and complete analysis.⁵⁻⁷ Recently, even the nano-mechanical behaviour of complex materials has been combined with different imaging techniques (SEM, TEM, EDS) to obtain, for the very first time, “correlated” information between structure, composition and functional and structural properties of the investigated materials, due to the nano-scale accuracy and positioning system of the involved equipment and the improvement given by coupling the graphical representation of the tested zone.⁸ The gap of knowledge is represented by the not completely understood correlations among chemical composition, microstructure and mechanical properties of nano-enabled materials, which need to be further investigated: furthermore, the fundamental mechanisms underlying the microstructure build-up and the in-service mechanical behaviour are still mostly unknown, due to the extreme complexity of such materials also with respect to the “representative volume element” (RVE) that must be harmonized between the various techniques.

The standard correlative light-electron microscopy approach is here empowered by the combination with other instruments (F.I.B., Nanoindenter and AFM) that were successfully employed in different applications such as cement based materials,⁹ lithium rechargeable batteries,¹⁰ MEMS,¹¹ residual stresses analysis¹² and more, integrating the unique features of each equipment and linking functional and dynamic information. The case studies presented will show how to couple the high-speed nanoindentation, the statistical deconvolution and SEM/EDS investigations to characterize the main phases present in the tested samples and to obtain high-resolution maps of the mechanical properties, in good agreement with SEM-EDS results; in addition even an innovative SEM-DIC combined technique for the residual stresses measurement on different materials is here reported.

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CURVATURE DRIVEN NANOPARTICLES DECORATION OF GRAPHENE MEMBRANES

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Graphene is extremely resilient to in-plane stresses because of its high Young's modulus¹; however, it can be easily bent to achieve complex folded structures, known as wrinkles². Such 3D out-of-plane deformations occur both naturally³ and in solution-chemistry processes⁴ and, as postulated in the literature, their curvature generate local potential centers with a short-range interaction⁵. The comprehension of the underlying mechanism beyond this phenomenon is fundamental for correlating the topographical properties of wrinkled graphene and its electronic structure. In order to investigate it, gold nanoparticles (NPs) were used to identify if and where they are pinned to wrinkles⁶. Here we report a morphological investigation of silver NPs coated by EG₆OH interacting with the surface of wrinkled graphene. The organic coating was used to reduce the metal-graphene interaction. A combination of Scanning Electron Microscopy (SEM) and Scanning Probe Microscopies (namely Atomic Force Microscopy – AFM – and Lateral Force Microscopy – LFM) was used to elucidate the early stage organization of NPs on wrinkled graphene. Firstly, Contact AFM and LFM were used to characterize the morphology of the graphene surface and its wrinkles, respectively. In particular, LFM was able to distinguish clearly three types of wrinkles, characterized by different folded structures⁷. Then, NPs were deposited on the graphene surface by drop casting, observing their organization on the surface by means of Tapping Mode AFM and SEM. Correlative images show that NPs diffuse on the graphene surface reaching wrinkles, then they diffuse along the topographic step formed by the wrinkle as long as a defect is met. The type of wrinkle seems not to affect this behavior.

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CORRELATIVE ELECTRON MICROSCOPY IN MODERN BIO-MEDICAL RESEARCH

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Correlative light–electron microscopy (CLEM) is a very effective technique that combines live-cell imaging and electron microscopy for ultrastructural morphological characterization of dynamic intracellular organelles.^{1,2} The use of fluorescent protein (FP)-tagged chimeras allows the user to follow the movements and/or behavior of intracellular structures in a live cell and to fix it at the moment of interest. The subsequent immuno-electron microscopy processing can then reveal the three-dimensional

architecture of the same structure, together with precise recognition of the FP-labeled protein.¹⁻³ The process resembles the taking of a high-resolution snapshot of an interesting live scene. The power of this approach opens new avenues for understanding complex cellular processes that operate in health and disease.

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3D HOLOTOMOGRAPHIC MICROSCOPY OPENS NEW ERA FOR LABEL-FREE LIVE CELL IMAGING

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Cellular analysis plays a crucial role in a wide variety of research and diagnostic activities in the life science. However, the information available to researchers and clinicians is limited by current microscopy techniques. An innovative new tool – holo-tomographic microscopy – can overcome many of these limitations and open new sights for researchers and clinicians to understand, diagnose and treat human diseases. Tomocube's HT technology¹ series utilize optical diffraction tomography (ODT), which enables users to quantitatively and noninvasively investigate live biological cells and thin tissues in 3D. Holo-tomography reconstructs the 3D refractive index (RI) distribution of live cells, that is an intrinsic optical parameter of a material and by doing so, provides structural and chemical information about the cell, including dry mass, morphology, and dynamics of the cellular membrane. We present an overview of possible applications by means of high-resolution optical tomographic images of live cells. Hematology², microbiology³, immunology⁴ and cell biology⁵ are the main fields of application. Quantitative and label-free imaging capability will open a new avenue for the study of pathophysiology of cells and tissues.

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NEW SOLUTIONS FOR CORRELATIVE MICROSCOPY

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There are many solutions available for correlative microscopy especially in electron microscopy. The functionality of the correlative software will depend greatly on the nature of the investigation and the variety of techniques being used for that investigation. Fortunately JEOL can offer several different products for correlation between scanning or transmission electron microscopes and multiple optical imaging technologies.

For TEM one problem is how to simply correlate sections on a grid for example with images from a diagnostic light microscope. In this case a simple image overlay option with guided automatic image alignment provides a quick and easy solution.

A similar approach can be used for SEM correlative microscopy and can provide the basis for simple high precision location of areas of interest, followed by multiple image and/or montage data acquisition on serial sections for example.

A more dedicated solution is also available using correlative specimen holders and software to enable specific site correlation between different experiments. In this case the workflow could include confocal and/or epifluorescence microscopy, sample cutting and high resolution field emission SEM imaging of the sites of interest.

This presentation will show some of the options available from JEOL which can offer both interactive and automated solutions for correlation between optical and electron microscopes.