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Libro degli abstract

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INDICE DELLE SESSIONI

PLENARY SPEAKERS

3D CHROMATIN FOLDING IN THE EYE OF THE STORM

Marie Victoire Neguembor

Reprogramming and Regeneration Lab, Center for Genomic Regulation (CRG), Barcelona, Spain

Understanding the complex interplay between genome folding and gene regulation is essential for unraveling the mechanisms underlying cellular functions. Here, we investigate the dynamic relationships among transcriptional activity and cohesin-mediated loop formation, and we develop modeling strategies to visualize gene folding with nucleosome resolution

Employing super-resolution microscopy techniques such as STORM and single molecule tracking, we examine how transcription-mediated supercoiling influences genome organization and the interaction between transcription and cohesin dynamics. Our findings reveal that increased cohesin loading and augmented looping, disrupt nuclear lamina integrity and lead to chromatin blending, ultimately influencing chromatin condensation and cohesin distribution. Altering supercoiling via either transcription or topoisomerase inhibition counteracts chromatin blending, increases chromatin condensation, disrupts loop formation, and leads to altered cohesin distribution and mobility on chromatin. Thus, we show that negative supercoiling generated by transcription is an important regulator of loop formation in vivo.

Additionally, we introduce an integrated imaging and computational approach, termed Modeling immuno-OligoSTORM (MiOS), which combines super-resolution STORM imaging with epigenomic data and molecular modeling. By integrating Hi-C and MNase-seq datasets, MiOS enables the modeling of genes at nucleosome resolution, providing insights into chromatin accessibility and gene conformation dynamics. With MiOS, we explore intercellular variability and transcription-dependent gene conformation in human pluripotent and differentiated cells, shedding light on the folding patterns of key genes associated with cellular identity and function.

Our study highlights the intricate relationship between genome architecture, transcriptional regulation, and chromatin dynamics. We offer new tools to investigate the principles governing nuclear organization.

A CLUSTER MODEL FOR THE TRANSFORMATION OF FIBROBLASTS TO MYOFIBROBLASTS

Farid Alisafaei[1,2], Tetsuro Wakatsuki[3], Guy Genin[1,4], Elliot L. Elson[5]

1 NSF Science and Technology Center for Engineering Mechanobiology
2 Department of Mechanical Engineering, New Jersey Institute of Technology
3 InvivoSciences. Inc., Madison WI
4 Department of Mechanical Engineering, Washington University in St. Louis
5 Department of Biochemistry and Molecular Biophysics, Washington University in St. Louis

The cells of solid organs such as the heart are embedded in a collagenous extracellular matrix (ECM) with biomechanical properties, e.g., viscoelastic stiffness, that enable the specialized organ-specific functions of the cells, such as cardiomyocyte contraction [1]. Under normal conditions fibroblasts maintain the required biomechanical properties of the ECM. Fibroblasts also can restore a wounded tissue by undergoing an epigenetic transformation to the myofibroblast phenotype, which shares properties of both fibroblasts and smooth muscle cells [2]. Myofibroblast contractility and collagen production repair tissue wounds, but also can have a pathological effect by stiffening tissues in ways that interfere with their normal functions (fibrosis). Central to model the transformation of fibroblasts to myofibroblasts is its promotion by stiffening of the ECM in which the cells are embedded [3]. Contractile force exerted by the cells increases matrix stiffness and this increased stiffness promotes transformation of fibroblasts to myofibroblasts. As the number of myofibroblasts increases, the overall contractile force they exert on the matrix increases correspondingly, further stiffening the matrix and thereby further accelerating the conversion of fibroblasts to myofibroblasts establishing a positive feedback loop as proposed in an earlier model for the fibroblast to myofibroblast transformation [4]. Our current analysis of this transformation recognizes that the stiffening is localized to the region of the ECM to which the cells are applying contractile force. Therefore, myofibroblasts are produced and accumulate in clusters. Additional clusters could be generated by the conversion of isolated fibroblasts to myofibroblasts at other tissue locations. This view is augmented by recent models of cell activation [5] and experimental observations of the formation of myofibroblast clusters in vitro and in vivo. This talk will describe our models for the transformation of fibroblasts to myofibroblasts within an ECM, focusing on the roles of matrix stiffening and formation of myofibroblast clusters.

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THE RACE TO UNDERSTAND INTELLIGENCE

Tomaso Poggio

Eugene McDermott Professor
Co-Director Center for Brains, Minds and Machines (CBMM)
Core founding scientific advisor, MIT Quest for Intelligence
McGovern Institute, CSAIL, Brain Sciences Department
M.I.T.

Will science or engineering win the race for AI? Do we need to understand the brain in order to build intelligent machines, or not? Are there theoretical principles underlying those architectures, including the human brain, that perform so well in learning tasks? Though we do not have a full theory of deep learning, there are good reasons to believe in the existence of some fundamental principles of learning and intelligence. In the race for intelligence, understanding such principles and applying them to brains and machines is a compelling and urgent need for our society.

THE SPY WHO CAME IN FROM THE COLD: UNDERSTANDING THE MECHANISM OF COLD DENATURATION

Annalisa Pastore

The Maurice Wohl Institute, King's College London, UK

FROM SUPER-RESOLUTION TO HIGH THROUGHPUT BRILLOUIN MICROSCOPY AT CLN²S

Giancarlo Ruocco

Center for Life Nano- and Neuro-Science (CLN2S) – Italian Institute of Technology (IIT), Director
Department of Physics) – Sapienza University of Rome, Professor.

First, I will briefly present an overview of the Rome's branch of the Italian Institute of Technology (IIT), the Center for Life Nano- and Neuro Science (CLN²S), including the more recent developments and the ongoing projects. These are mostly oriented toward the development of new technological tools for biological imaging, tissue engineering, iPSC expansion, drug delivery, etc. Particularly attention will be devoted to the high throughput Brillouin (i.e. biomechanical) microscopy, to animal-based cancer biosensors, and to a new super-resolution microscopy technique capable of the early diagnosis of neurodegenerative diseases.
The latter is a technique allowing to reach a very high spatial resolution (150 nm) with very long working distance (25 mm) objectives. This allow to look at the retinas of humans *in-vivo*, in a non-invasive manner, and to search for the presence of those protein aggregations that are the landmark of many neurodegenerative diseases. Our studies on the retinas of animal models (mutant mice recapitulating the Alzheimer Disease, AD) and on post-mortem AD human patients (obtained by eye- and brain-banks) shown that the same progression of the pathology takes place in the brain and in the retina, and that the observation of 200 nm sized betaAmyloid and TAU proteins aggregates would anticipate by 15/20 years the first AD symptoms. The potential applications of this instrument encompass a wide spectrum, ranging from its utilization in mass screening in the long term to serving as a fundamental tool for pharmaceutical companies seeking to expedite the evaluation of trial drugs for Alzheimer's disease therapy in the short term.

THE PHASOR APPROACH FOR BIOPHYSICAL STUDIES OF CONDENSATES AND MEMBRANES USING DAN PROBES.

Leonel Malacrida

Institut Pasteur de Montevideo, Montevideo, Uruguay

The phasor approach has become a powerful method for quantitatively analyzing FLIM and HSI data without relying on specific models. Its straightforward rules and user-friendly nature make phasor plots accessible to non-experts in these fields. Utilizing these tools, we investigate the biophysical properties of cellular interiors with dimethylamino-naphthalene probes, which offer insights into molecular crowding and water dynamics. In this presentation, I will discuss our recent research on how these tools enhance our understanding of the role of condensates in membrane dynamics and organization. Additionally, I will introduce our group's latest development: PhasorPy, an open-source Python library designed for comprehensive phasor analysis.

ENDO- / LYSOSOMAL CLC TRANSPORTERS: FROM BIOPHYSICS TO HUMAN NEUROLOGICAL DISEASES

Michael Pusch

Istituto di Biofisica, CNR, Via De Marini 6, 16149 Genova, Italy, michael.pusch@ibf.cnr.it

Intracellular endolysosomal trafficking regulates important cellular processes. Defects in endolysosomal processing lead to a large number of human diseases. These affect predominantly neurons, likely because their post-mitotic nature leads to accumulation of damage over years. For example, Alzheimer's disease has been associated with endosome dysfunction. Five members of the gene family of voltage-gated ChLoride Channels (CLC proteins) are localized to endolysosomal membranes, where they carry out anion/proton exchange activity and thereby regulate pH and Cl^- concentration. Mutations in these vesicular CLCs cause global developmental delay, intellectual disability, various psychiatric conditions, lysosomal storage diseases, and neurodegeneration, resulting in severe pathologies or even death.
Our group has been involved in studying human diseases related to CLC-3[1], CLC-4[2], CLC-6[3,4], and CLC-7[4,5]. In CLC-3 and CLC-4 we investigated a large number of missense variants found in pediatric patients and discovered two novel, probably dominant disease mechanisms that are related to CLC oligomerization. Some disease variants affect the voltage-dependent activation mechanism of CLC-4, causing essentially a loss of function. Other variants instead disrupt the gating process, leading to constitutively active transporters, a toxic gain of function effect. Interestingly, many of these "gate-altering" variants are localized at the subunit interface of the homodimeric CLC proteins, suggesting that gating of CLC transporters involves a rearrangement of the interface. Measuring Förster resonance energy transfer (FRET) using fluorescence lifetime imaging microscopy (FLIM-FRET) between GFP-tagged CLC-3 and mCherry-tagged CLC-4 we could demonstrate close interaction of the two proteins. The method will be employed to test whether disease-associated CLC-4 or CLC-3 variants impact the dimerization process.
Finally we discovered that the lysosomal CLC-7 transporter is inhibited by extracellular / luminal Cl^-, while the late endosomal CLC-6 is activated by Cl^-[4].The opposite dependence of CLC-6 and CLC-7 on their transported substrates suggests non-overlapping physiological roles. The inhibition of CLC-7 by luminal Cl^- likely serves to limit excessive intraluminal Cl^- accumulation, thereby preventing osmotic overload of lysosomes.

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BRILLOUIN MICROSCOPY FOR CELL AND TISSUE IMAGING

Giuliano Scarcelli

University of Maryland, College Park (USA)

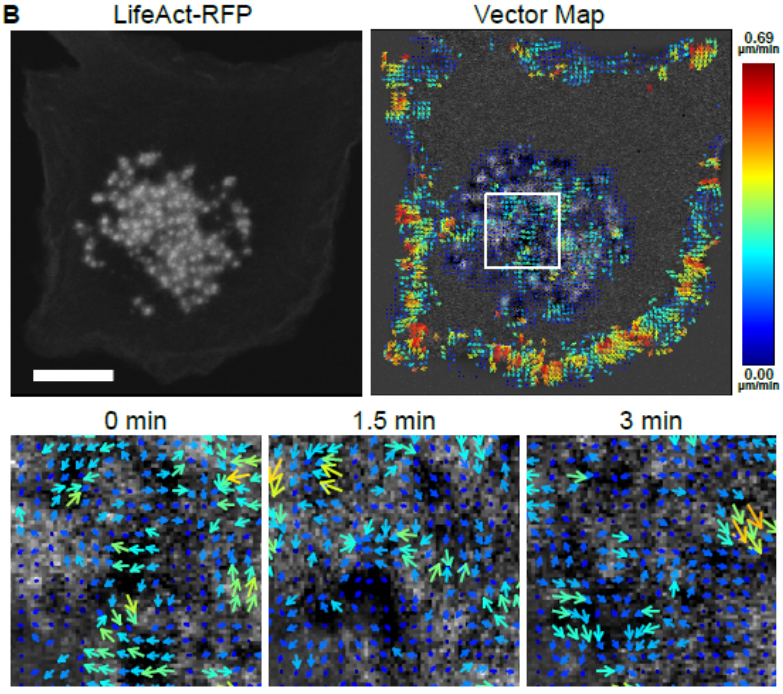
The interaction between photons and acoustic phonons within materials, first described by Leon Brillouin, has been widely investigated to characterize mechanical and physical properties of samples. To translate this technology to biomedical applications where mechanical properties are often critical, our lab has developed high-resolution spectrometers at high throughput and combined them with optical microscopes to yield 3D imaging modalities that use label-free biophysical properties as contrast mechanisms for imaging. Our first area of application has been in ophthalmology as the loss of corneal strength leads to ectasia and is a major risk factor for refractive surgery complications. To address this issue, we have developed an in vivo Brillouin ophthalmoscope and encouraging data show we can differentiate ectatic corneas as well as characterize outcome of emerging treatments. Recently we have demonstrated increased Brillouin microscopy resolution to characterize intracellular modulus and we developed a flow cytometry platform to rapidly characterize cells based on their mechanical properties. Finally, we will discuss the physics behind the interpretation of Brillouin spectral signatures in the context of soft matter such as cells and tissues.

DECIPHERING THE OSCILLATORY DYNAMICS OF PODOSOMES IN HUMAN DENDRITIC CELLS USING IMAGE CORRELATION METHODS

Paul W. Wiseman

Department of Physics and Department of Chemistry, McGill University, Montréal, Canada

Podosomes are punctate actin-rich cytoskeletal structures used by dendritic cells and macrophages to sense the mechanical properties of the surrounding extracellular environment, helping them patrol for pathogens. Podosomes are sensitive to substrate stiffness and probe the extra-cellular matrix by polymerizing actin from the cap against the plasma membrane, exerting force onto the substrate and detecting the opposing forces. Cycles of polymerization and collapse result in height oscillations of the podosome core. Interestingly, podosomes form clusters in cells that exhibit collective oscillatory behavior, resulting in podosome height waves in which a local group of podosomes sequentially increase and then decrease their F-actin content. Previously, we have studied the oscillatory dynamics in podosomes using image correlation analysis of fluorescence microscopy image time series. Image correlation methods are an extension of fluorescence fluctuation spectroscopy that can measure protein-protein interactions and macromolecular transport properties from input fluorescence microscopy images of living cells. These approaches are based on space and time correlation analysis of fluctuations in fluorescence intensity within images recorded as a time series using a fluorescence or super-resolution microscope. I will introduce spatio-temporal image correlation spectroscopy (STICS) and its 2-color cross-correlation variant (STICCS) and describe the application of the image correlation methods for characterizing intensity waves of talin, vinculin as well as filamentous actin imaged via confocal and Airy scan super-resolution fluorescence microscopy for networks of assembling and disassembling podosomes in human dendritic immune cells. I will briefly introduce a new biophysical model that recapitulates the collective podosome dynamics and focus on the application of STICS on both simulated and experimentally measured podosome wave dynamics within cells under different treatment perturbations to validate the theoretical model.



Actin waves revealed by STICS in a podosome cluster in immune dendritic cells

WHEN BIOPHYSICS MEETS BIOCHEMISTRY: THE MECHANISMS OF CANCER CELL MICRONUCLEI COLLAPSE UNVEILED

Melody Di Bona,[1] Yanyang Chen,[1] Albert S. Agustinus[1,2], Matthew Deyell,[2] Lorenzo Scipioni,[3] Sara Martin[4], Stefano Santaguida[4], Benjamin Izar[5], Enrico Gratton[3], Alban Ordureau[1], Ashley Laughney[2], John Maciejowski,[1] Thomas Jeitner[2] and Samuel F. Bakhoum[1]

[1]Memorial Sloan Kettering Cancer Center, 1027 York Avenue, 10065 New York, NY, USA
[2]Weill Cornell Medicine, 1305 York Avenue, 10065 New York, NY, USA
3LFD, University of California, 3208 Natural Sciences II, 92617 Irvine, CA, USA
4IEO, Via Ripamonti 435, 20141 Milano, Italy
[5]Columbia University, 116th and Broadway, 10027 New York, NY, USA
dibonam@mskcc.org

Chromosomal instability is a hallmark of most human cancers, and is related to therapy resistance, distant metastasis and poor prognosis. Chromosomally unstable cancer cells are characterized by micronuclei, aberrant organelles containing missegregated chromosomes. Micronuclei often undergo irreversible rupture, exposing their DNA to the cytosol where it activates pro-metastatic pathways[1] and catalyzes extensive heritable genomic[2] and epigenetic rearrangements[3, 4]. Micronuclear collapse is thus a central event for tumor evolution and metastatic progression[5], and its consequences have been linked to poor-prognosis and therapy resistance; nonetheless, the mechanisms driving micronuclear rupture are still obscure. Here, thanks to the pioneering integration of biophysics methodologies and insights into cancer research, we unveiled a decades-long mystery, illuminating the intricate mechanisms underlying micronuclear collapse with unprecedented clarity and emphasizing the indispensable role of these approaches in unraveling the elaborate complexities of tumorigenesis. In fact, we identified the local high concentration gradient of reactive oxygen species (ROS) at the mitochondrial production site as the main cause of micronuclei collapse. Notably, through super-resolution imaging and 3D-volume reconstruction, we observed that micronuclei located closer to mitochondria are more prone to rupture. Accordingly, experimentally increasing ROS incremented rupture, while decreasing ROS reduced the frequency of ruptured micronuclei. By using a combination of advanced super-resolution microscopy, spectroscopy, proteomics, cell biology techniques, in vitro biochemistry assays, and extensive mutagenesis, we revealed the exact pathway leading to micronuclei collapse and we demonstrate that ROS promote a noncanonical function of the membrane repair ESCRT-III complex scaffolding protein, CHMP7. ROS inhibit the exportin XPO1, impairing CHMP7 micronuclear export and subsequently driving its accumulation. Furthermore, ROS reduce CHMP7 interaction with ESCRT-III, promoting CHMP7 oligomerization and its binding to the inner nuclear membrane protein, LEMD2. CHMP7, while aggregating, physically pulls the micronuclear envelope together with the LEMD2-associated lamina, thereby disrupting micronuclear integrity. Finally, we show that hypoxic conditions promote ROS-dependent CHMP7-LEMD2 interaction, inducing micronuclear rupture and inflammation. Thus, we observe that hypoxic human tumors have a significantly increased predominance of ruptured micronuclei, providing a mechanistic link between tumor hypoxia and downstream processes that drive cancer progression.It is only through the integration of biophysics with cancer biology that this study was able to expand our understanding of tumor aggressiveness and, by providing new putative therapeutic targets, to pave the way for the development of more effective treatments. By deciphering the intricate molecular players orchestrating cancer progression, this research offers newfound hope in the quest for improved therapies, ultimately poised to transform the landscape of cancer treatment.

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TISSUE FLUIDIFICATION IN PATHOPHYSIOLOGY

Giorgio Scita

Department of Oncology and Hematology-Oncology, Università degli Studi di Milano, Milano

The process in which locally confined epithelial malignancies progressively evolve to become invasive cancer cells is associated with the acquisition of cell motility, fostered by a mechanical shift from a solid-like to a liquid-like state, known as unjamming. The biomolecular machinery behind unjamming has only begun to be unraveled, while whether the perturbations associated with this tissue-level mechanical transition impact on phenotypes during carcinoma progression remains unclear. Using a combination of physical approaches, ex vivo and in vivo model systems, we will address these issues and discuss whether an endocytic - driven transition between “solid” and “liquid” states of cell collectives is a complementary gateway to cell migration in pathology, focusing specifically on the progression of early breast cancer lesions that become locally invasive. We will show how these dynamics changes featuring the coexistence of long-range coordinated motion and local cell re-arrangement are sufficient to promote matrix remodeling, and local invasion and exert mechanical stress on individual cell nuclei. This is accompanied by profound transcriptional rewiring, with the unexpected activation of an inflammatory response, change in cell state, and the emergence of malignant traits.

INVITED SPEAKERS

LIPID MEMBRANE REMODELLING AND MEMBRANE FUSION INDUCED BY SYNTHETIC NANOPARTICLES

Giulia Rossi

Department of Physics, University of Genoa, Genoa, Italy

Membrane fusion is essential for the basal functionality of eukaryotic cells. In physiological conditions, fusion events are regulated by a wide range of specialized proteins, operating with finely tuned local lipid composition and ionic environment. Fusogenic proteins, assisted by membrane cholesterol and calcium ions, provide the mechanical energy necessary to achieve vesicle fusion in neuromediator release. Similar cooperative effects must be explored when considering synthetic approaches for controlled membrane fusion. Here we show that liposomes decorated with amphiphilic Au nanoparticles (AuLips) can act as minimal tunable fusion machinery. By means of coarse-grained Molecular Dynamics simulations, we address the mechanisms of fusion and highlight how the nanoparticle size and the membrane curvature can determine the stability of fusion intermediates. The results provide a novel contribution to developing new artificial fusogenic agents, and contribute to the fundamental understanding of the physical forces driving fusion events.

CHALLENGES AND OPPORTUNITIES IN MD SIMULATIONS OF BIOSYSTEMS: FROM PROTEINS TO CELLS?

Gianluca Lattanzi

Dipartimento di Fisica, Università di Trento, Trento

Molecular simulations have emerged as a reliable tool to investigate protein structure, dynamics, and function, as well as their interactions with other biomolecules. In particular, molecular dynamics (MD) simulations stand out due to their ever increasing accuracy and ability to sample conformational states with reliable statistical significance. In this talk, I will showcase some recent challenging applications of MD simulations in which computational methods are tailored to address specific research problems, complemented by experimental data that are always necessary to validate computational results. As we look towards the future, I will discuss the incoming challenges in the field: what are the next steps? How far are we from the simulation of an entire cell?

ANTIBACTERIAL PHOTOKILLING: LIGHT-BIOFILM INTERACTION MODELS OF INCREASING COMPLEXITY

Giovanni Romano,[1] Giacomo Insero,[1] Franco Fusi,[1] Lucia Pallecchi,[2] Maria Micieli,[3] Eleonora Riccobono,[2] Nidia Maldonado-Carmona,[4,1] Nelly Henry,[4] Murielle Balthazar,[5] Johanne L Fothergill,[5] Aras Kadioglu[5]

[1]Department of Experimental and Clinical Biomedical Sciences, University of Florence, Viale Pieraccini 6, 50139 Firenze, Italy
[2] Department of Medical Biotechnologies, University of Siena, Viale Bracci 2, 53100 Siena, Italy
[3] Department of Experimental and Clinical Medicine, University of Florence, Largo Brambilla 3, 50134 Firenze, Italy
[4] Sorbonne Université, CNRS, Laboratoire Jean Perrin, Place Jussieu 4, Paris, France
[5] Department of Clinical Infection Microbiology and Immunology, University of Liverpool, Liverpool, UK
giovanni.romano@unifi.it

In antibacterial photoinactivation, both *in vitro* and *in vivo* biofilm models are long developed in view of clinical applications. Different illumination geometries and settings have been explored, aiming at studying the treatment efficacy dependence on both physical (light-related), chemical (photosensitizer / enhancer) and biological (e. g. strain) factors.
To have a more comprehensive vision of the biofilm response to photoinactivation, experiments will be presented with different biofilm type and geometry, in the case of two of the most diffused bacterial species responsible for lung chronic infections: *Pseudomonas aeruginosa* (PAO1, LESB65) and *Staphylococcus aureus* (USA300, MRSA). In a first "macroscopic" approach, both peg-adherent biofilms and planar growing biofilm models were irradiated by LED sources peaked at 405, 445, 525 and 623 nm, to acknowledge the photokilling efficacy dependence on both the irradiation wavelength and dose, exploiting the presence of endogenous photosensitizing porphyrins. The results in terms of post-irradiation CFU counting were confronted with the outcome of representative experiments where a "microscopic" approach was considered, based on a growing biofilm model studied by milli-fluidic and optical video-microscopy techniques.[1] In this case, the photoinactivation efficacy of 405 nm light was measured by following the bacterial population density in time, thanks to optical density and fluorescence measurements. Furthermore, a theoretical model was developed to mimic the biofilm response to photo-inactivating light, inferring it from cell photokilling models developed in radiobiology.[2,3] Experimental data fitting with the model prediction allowed for the estimation of the bacteria photosensitivity, proposing the existence of a coupled physical and biological response of the biofilm itself.
Finally, preliminary *ex vivo* experiments with infected mouse models will be presented, to analyze the influence of a complex tissue structure on the photokilling outcome. In this case, 405 and 528 nm laser irradiation was performed post mouse infection on *ex vivo* lungs.

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BIOMOLECULES ON SURFACES: FUNCTIONAL INTERFACES FOR SENSING APPLICATIONS

Ornella Cavalleri

Dipartimento di Fisica, Università di Genova, Via Dodecaneso 33, 16146 Genova, Italy
ornella.cavalleri@unige.it

The self-assembly of biomolecules on surfaces has played a pivotal role in the development of functional interfaces for biosensing purposes. A detailed control of the molecular organization at the interface is a key requirement for the design of reliable and reproducible sensors. In the last years, we have developed a multi-technique analytical approach to evaluate the sensing properties of hybrid interfaces by coupling Atomic Force Microscopy (AFM), Spectroscopic Ellipsometry (SE), X-ray photoemission spectroscopy (XPS), and Quartz Crystal Microbalance with Dissipation (QCM-D) analysis. We studied self-assembled monolayers (SAMs) of thiol terminated molecules on gold, from simple alkanethiols to DNA strands and proteins[1-6]. The combined use of multiple analytical methods, which probe different interfacial properties, is particularly effective for characterizing ultrathin molecular films, as it increases the degree of confidence in the obtained results. We will focus on a label-free detection platform for the recognition of oligonucleotide target sequences based on hybridization of surface immobilized probe DNA strands. Combining SE and QCM-D enabled quantification of the assay and demonstrated the selectivity and recovery properties of the sensing platform. A detailed optical model, supported by information on film thickness and surface composition, allowed to identify UV DNA resonances and DNA hypochromism at the single molecular level. To proceed toward a multiplex targeting of different sequences we exploited AFM nanolithography to graft platforms of different DNA probe sequences with tunable surface density for optimizing the hybridization efficiency with target sequences. The method, applied to the RNA-dependent RNA polymerase /helicase sequence (RdRp/Hel) of SARS-CoV2, allowed to discriminate between the RdRp/Hel sequences of different Corona Viruses.

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THE MECHANICAL AND ADHESIVE PROPERTIES OF CELLS AND TISSUES IN HEALTH AND DISEASE INVESTIGATED BY ATOMIC FORCE MICROSCOPY

Alessandro Podestà

Dipartimento di Fisica "Aldo Pontremoli",
Università degli Studi di Milano, Milano, Italy
e-mail: alessandro.podesta@unimi.it

The role of forces is fundamental in several biological processes. Cells can perceive physical stimuli from the surrounding microenvironment (the extracellular matrix, ECM) through extremely complex processes mediated by several different molecular machineries. Unravelling which peculiar properties of the ECM determine specific cellular responses, understanding the mechano- sensing processes and how cells, in turn, modify the physical state of the microenvironment in relation to changes of the physio-pathological state of the tissue, could help elaborating diagnostic approaches based on mechanical quantitative nanoscale measurements. Here we describe techniques based on Atomic Force Microscopy (AFM) for the characterization of structural and mechanical properties of cells, tissues, biomembranes and their interactions with the microenvironment. These approaches include nanomechanical (nanoindentation) as well as adhesion force spectroscopy measurements. The capability of AFM to investigate systems of biological and clinical relevance is demonstrated through some examples taken from recent studies. See also:

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BIOPHYSICAL TOOLS FOR ADVANCED RADIOBIOLOGY STUDIES IN VITRO

Vittorio Parenti[1], Benedetta Noferi[1], Claudia Martini[2,3,4], Martina de Felice[1,2], Francesca del Debbio[2], Eleonora da Pozzo[2,3,4], Raffaella Magrassi[5,6], Simone Lossano[1,7], Retico[7], Simone Capaccioli[1,3,4] and Francesca Cella Zanacchi[1,3,5]

[1]Department of Physics E. Fermi, University of Pisa, Largo Bruno Pontecorvo, 56127 Pisa, Italy
[2] Department of Pharmacy, University of Pisa, Via Bonanno Pisano, 12, 56126 Pisa, Italy
[3]Center for Instrumental Sharing of the University of Pisa (CISUP), Pisa, Italy
[4] Centro Pisano Multidisciplinare Sulla Ricerca e Implementazione Clinica della FLASH Radiotherapy (CPFR), University of Pisa,
Presidio Santa Chiara, ed.18 Via Roma 67, 56126 Pisa, Italy
[5] Nanoscopy and NIC@IIT, Istituto Italiano di Tecnologia, 16152 Genoa, Italy
[6] Istituto di Biofisica, Research National Council (CNR) Genoa, Italy
[7] National Institute for Nuclear Physics (INFN) – Pisa Section, Via Filippo Buonarroti, 3, 56127 Pisa, Italy

The joint effort of optics, photonics, spectroscopy and chemistry gave rise to a new family of fluorescence imaging techniques, able to overcome the diffraction barrier by pushing the resolution down to tens of nanometers. In particular, super-resolution microscopy (SR) based on single molecule localization, originally driven by pushing the resolution limit, is becoming an effective tool for quantitative biological studies. We will focus on the role of biophysical tools, such as cutting-edge fluorescence imaging technologies and Artificial intelligence approaches, in highlighting the molecular and sub-cellular mechanisms underlying the response to ionizing radiations.
In particular, we will use quantitative super-resolution microscopy [1] to elucidate in vitro the effects induced by the different irradiation conditions and regimes, focusing on different radiobiological endpoints of interests. We show the ability of super-resolution to target proteins distribution associated with different radiobiological endpoints, ranging from DNA damage and repair proteins to cytoskeletal proteins.
The presented biophysical approaches have the potential to provide innovative insights within the radiobiology context elucidating the so-called Flash effect.
Among the cutting-edge radiotherapy techniques, FLASH radiotherapy stands out as a promising tool able to revolutionize the conventional radiotherapy approaches. In FLASH-RT an equivalent dose is reached delivering ultra-high dose rates in high intensity short pulses. This irradiation strategy apparently preserves the same efficacy of conventional radiotherapy on cancer treatment while selectively spare normal tissues [2,3], thus providing the so-called FLASH effect. Still, the full understanding of the FLASH effect and its molecular mechanisms remain largely unclear [4]. In this scenario a first detailed comprehension of the FLASH-related molecular mechanisms is needed and may be accessed studying *in vitro* the molecular biomarkers involved by means of quantitative super-resolution microscopy.

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SUPER-RESOLUTION THERMAL IMAGING: METHODS AND APPLICATIONS

Maddalena Collini

Dipartimento di Fisica, Università degli Studi di Milano-Bicocca, Piazza della Scienza 3, 20126 Milano, Italy

Super-resolution in optical microscopy has gained popularity in biological applications, offering new insights into cellular processes and nanoscale interactions. Extending this concept to the far-infrared range, we introduce a label-free, photo-activated thermal imaging technique that employs super-resolution for high-sensitivity, high-resolution temperature-based imaging of biological samples, from tissue scale to single-cell level.

We developed two approaches that utilize structured illumination to promote sample heating with focused visible laser light. The distribution of laser-induced temperature variations is detected by a microbolometer thermal camera. By localizing hot spots or iteratively modeling the 3D heat diffusion, we achieve image reconstruction at micrometer-scale spatial resolution, with up to a 200-fold enhancement over the thermally-diffused point-spread-function of the same camera under standard operation over the seconds timescale1.

We demonstrate the efficacy of this technique in several biophysical applications, including mapping the spatial distribution of Perls' Prussian Blue (PB)-stained hepatic iron deposits associated with hereditary hemochromatosis in human liver biopsy specimens, and quantifying the molar concentrations of melanin pigments in excised murine melanoma biopsies with tens of microns of spatial resolution2.

Additionally, we applied this technique to material science and cultural heritage conservation by mapping thermal conductivity at the mesoscopic scale3. This novel super-resolution thermal imaging technique shows great promise for the thermal characterization of a wide range of materials.

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ONCE IN A (FLUORESCENCE) LIFETIME: FROM SYNTHETIC IDENTITY TO BIOLOGICAL FUNCTION OF NANOENCAPSULATED DRUGS IN BIOMEDICAL APPLICATIONS

Francesco Cardarelli

NEST Laboratory - Scuola Normale Superiore, Piazza San Silvestro 12, Pisa, Italy.

Fluorescence Lifetime Imaging Microscopy (FLIM) is an established tool to address molecular questions in vitro and in vivo, due to its exquisite sensitivity to the nanoscale environment of the emitter. Popular applications range from the study of molecular interactions to bio-sensing, from the measurement of the intrinsic live-cell/-tissue autofluorescence lifetime to its interpretation in terms of metabolic switch, differentiation, progression to a pathological state, etc. Based on this background, here we pursue an innovative line of research on nano-encapsulated compounds (e.g. drugs) and their biomedical application which builds on FLIM nanoscale sensitivity. By exploiting the intrinsic fluorescence signal of an encapsulated compound, FLIM is used to quantitatively extract information on its supramolecular organization within, for instance, a pharmaceutical formulation, from the production phase to the application in living matter. DOXIL®, the liposomal formulation of the well-known anticancer drug Doxorubicin, is presented as a first case study. By the fit-free, fully-graphical phasor-FLIM analysis, the signature of DOXIL® is first resolved into the contribution of three co-existing drug species, each with its characteristic mono-exponential lifetime, namely: crystallized drug, free drug, and drug bound to the liposomal membrane. Then, the exact molar fractions of these three species are determined by combining phasor-FLIM with quantitative absorption/fluorescence spectroscopy on pure standards. Finally, preliminary data on the application of DOXIL® in living cells are presented and interpreted in terms of time evolution of drug bioavailability and supramolecular state. To prove the potency of the proposed approach, additional nano-encapsulated compounds are disccussed alongside their application in different fields of nanomedicine, from the treatment of Diabetes to neurodegenerative diseases, from cancer to metabolic disorders.

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INTERPLAY BETWEEN BIOMOLECULAR CONDENSATES AND AMYLOID FORMATION

Paolo Arosio

Department of Chemistry and Applied Sciences, ETH Zurich, Zurich, Switzerland

It is emerging that cells can coordinate biochemical activity in space and time via membraneless organelles formed by phase separation of proteins and nucleic acids. In some cases, these organelles, also known as biomolecular condensates, can age over time into amyloid fibrils, a process which has been associated with neurodegenerative diseases. Here, we present molecular mechanisms that regulate the formation of fibrils from multicomponent biomolecular condensates. We show effects that go beyond the local increase of protein concentration due to phase separation. In particular, we demonstrate the effect of interface[1][2] and heterotypic interactions[3] on amyloid formation mediated by condensation. We focus on the formation of amyloids from biomolecular condensates of hnRNPA1, a protein involved in Amyotrophic Lateral Sclerosis (ALS).[1][2]

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NANOSCALE ORGANIZATION OF PROTEINS AS CRUCIAL MODULATOR OF CELL'S PATHWAYS

Barbara Storti

NEST, Scuola Normale Superiore and Istituto Nanoscienze-CNR, Piazza San Silvestro 12, 56127 Pisa, Italy

Biomolecular condensates are found throughout eukaryotic cells, including in the nucleus and on membranes. The function of this nano and mesoscale cellular organization moves from the concentration to the exclusion of biomolecules for modulating the activity of proteins and other biomolecules. In the talk, I will discuss representative examples of biomolecular condensates by using quantitative fluorescence microscopy leveraging a toolbox of high spatio-temporal resolution techniques such as FRET, Fluorescence correlation spectroscopy, as well as nanoscopy approaches. Such toolbox enables monitoring the specific dynamics, clustering status and interaction interplay of membrane and nuclear condensates.

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BIOFISICA TEORICA E COMPUTAZIONALE

MOLECULAR INSIGHTS INTO THE RESCUE MECHANISM OF AN HERG ACTIVATOR AGAINST SEVERE LQT2 MUTATIONS.

Amit Kumawat[1], Elisa Tavazzani[2,3], Giovanni Lentini[4], Alessandro Trancuccio[2,3], Deni Kukavica[2,3], Silvia G. Priori[2,3,5], and Carlo Camilloni[1]

1Department of Biosciences, University of Milano, Milano, Italy
2Istituti Clinici Scientifici Maugeri Research Hospital (IRCCS), Pavia, Italy
3Molecular Cardiology, Department of Molecular Medicine, University of Pavia, Pavia, Italy
4Department of Pharmacy-Pharmaceutical Sciences, University of Bari Aldo Moro, Bari, Italy
5Centro Nacional de Investigaciones Cardiovasculares Carlos III, Madrid, Spain
carlo.camilloni@unimi.it

Mutations in the hERG potassium channel are a major cause of long QT syndrome type 2 (LQT2), which can lead to sudden cardiac death. The hERG channel plays a critical role in the repolarization of the myocardial action potential, and loss-of-function mutations prolong cardiac repolarization. In this study, we investigated the efficacy and mechanism of ICA-105574, an hERG activator, in shortening the duration of cardiac repolarization in severe LQT2 variants. We characterized the in vivo efficacy of ICA-105574 in shortening the QT duration in an animal model and the in vitro I_{Kr} current in cellular models mimicking severe hERG channel mutations (A561V, G628S, and L779P). We then used molecular dynamics simulations to investigate the molecular mechanisms of ICA-105574 action. In vivo, ICA-105574 significantly shortened the QT interval. LQT2 mutations drastically reduce I_{Kr} amplitude and suppress tail currents in cellular models. ICA-105574 restores I_{Kr} in A561V and G628S. Finally, in silico data showed that ICA-105574 stabilizes a pattern of interactions similar to gain-of-function SQT1 mutations and can reverse the G628S modifications, through an allosteric network linking the binding site to the selectivity filter and the S5P turret helix, thereby restoring its K^+ ion permeability. Our results support the development of hERG activators as pharmacological molecules against some severe LQT2 mutations and suggest that molecular dynamics simulations can be used to test the ability of molecules to modulate hERG function in silico, paving the way for the rational design of new hERG activators.

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COMPUTATIONAL STRATEGIES FOR PROBING PROTEIN INTERACTIONS: PREDICTIVE INSIGHTS INTO INTERACTING RESIDUES

Greta Grassmann,[1,2], Lorenzo di Rienzo,[2] Giancarlo Ruocco,[1,2] Mattia Miotto,[2] Edoardo Milanetti[1,2]

[1]Sapienza University of Rome, Piazzale Aldo Moro 5, 00185, Rome, Italy 2Istituto Italiano di Tecnologia, Viale Regina Elena 291, 00161, Rome, Italy
greta.grassmann@uniroma1.it

Understanding and predicting protein-protein interactions (PPIs) is crucial, given that over 80% of proteins function within molecular complexes that are central to biological processes.
Comprehending their formation can provide crucial information on the physiological and pathological cellular life. Cells are crowded with molecules and other components, among which proteins must recognize and adapt to bind, making complex formation highly specific. This compatibility is determined by an interplay between various contributions on the molecular surface: once the proteins are bound, their binding regions display a combination of geometrical and chemical complementarities, which ultimately reflect on the binding stability.
Computational methods offer insight into single-molecule behaviors in controlled settings, aiding experimental studies to uncover these mechanisms.
In this context, we already proposed an algorithm based on the evaluation of the shape complementarity at the interfaces using the Zernike 2D polynomial expansion, which was able to identify the real interacting regions in ~60% of the analyzed cases [1]. These numerical descriptors are invariant under rotation, allowing a quick and easy comparison between regions of different proteins during the blind earch for interacting patches.
In principle the Zernike formalism could be applied to any property, besides shape, that can be described with numerical values assigned to each surface point. By expanding the protocol to assess electrostatic complementarity, we were able to discriminate between transient and permanent PPIs with a ROC AUC of 0.8 [2]. Preliminary tests employing a neural network integrating geometric and chemical descriptors, and the aforementioned features, achieved an accuracy of around 0.8 in identifying interacting residues.
The resulting protocol could be employed to reinforce docking or pose selection algorithms, as well as to favor the development of new predictive methods for binding affinity.

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MANIPULATING PROTEIN ORIENTATION WITH STRONG ELECTRIC FIELDS: MOLECULAR DYNAMICS SIMULATIONS FOR INSIGHTS INTO SINGLE PARTICLE IMAGING AT X-RAY FREE-ELECTRON LASERS

Emiliano De Santis,[1,2,3] *Maxim Brodmerkel*,[1] *August Wollter*,[4] *Erik Marklund*,[1] *and Carl Caleman*[2,5]

[1] Department of Chemistry – BMC, Uppsala University, Box 576, SE-751 23 Uppsala, Sweden
[2] Department of Physics and Astronomy, Uppsala University, Box 516, SE-751 20 Uppsala, Sweden
[3] University of Rome Tor Vergata & INFN, Rome, 00133, Italy
[4] Department of Cell and Molecular Biology, Laboratory of Molecular Biophysics, Husargatan 3, 75124, Uppsala, Sweden
[5] Center for Free-Electron Laser Science CFEL, Deutsches Elektronen-Synchrotron DESY, Notkestraße 85, DE-22607 Hamburg, Germany
emiliano.desantis@physics.uu.se

Single particle imaging is a set of emerging techniques that utilize ultrashort and ultraintense X-ray pulses to generate diffraction from single isolated particles in the gas phase to determine their structures.[1] However, one of the main challenges of this approach is the unknown orientation of the individual sample molecules at the time of exposure, which makes structure determination computationally demanding and, in some cases, impossible. Proteins often have nonzero electric dipole moments, making them interact with external electric fields and offering a means for controlling their orientation[2].
Through classical and ab initio molecular dynamics simulations, we identify a range of electric field strengths that allow for successful orientation of proteins without altering their structure.[3,4,5] Additionally, we demonstrate that orientation information is crucial for successful structure determination in various experimentally relevant cases.[6] Our findings suggest that non-destructive field orientation of intact proteins is a viable method that opens up new avenues for structural investigations using single particle imaging.

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COMPUTATIONAL INVESTIGATIONS FOR THE DESIGN OF A MULTIMODAL INNOVATIVE THERANOSTIC NANOSYSTEM (MITHOS)

Julia Bassila,[1] Davide Bochicchio,[1] Sonia Cambiaso,[1] Enrico Lavagna,[1] and Giulia Rossi [1]

[1]University of Genoa - Physics Department, Via Dodecaneso, 33, 16146 Genova GE, Italy
julia.bassila@edu.unige.it

Drug delivery systems based on stimuli-responsive metal oxide nanoparticles (MeOx NPs) for cancer treatment gained interest in the past few years. Due to their enhanced targeting ability, they can limit the drugs toxic effects and allow for better tuning of the dose/response behavior1,2. Biomedical MeOx NPs rely on NP surface functionalization with different agents, which allows their solubility, drug loading and specific targeting. Within the PRIN project MITHoS, functionalized NP are covered by a lipid membrane and used as an ultrasound-responsive carrier of the anti-cancer drug carfilzomib (CFZ) against multiple myeloma. Here, we used Molecular Dynamics (MD) simulations to investigate the structure and dynamics of these functionalized MeOx NPs and guide the design of better-performing theranostic agents. Our target NP is a ZnO NP functionalized with oleic acid (OLA) and 3-(aminopropyl)trimethoxysilane (APTMES) or N-(2-Aminoethyl)-3-aminopropyltrimethoxysilane (l-APTMES). We designed molecular models with a coarse-grained (CG) resolution of ZnO NPs, ligands, and CFZ drug. We performed metadynamics simulations to characterize CFZ adsorption on a ZnO surface functionalized with APTMES and l-APTMES in water and in ethanol. The free energies of adsorption profiles showed that using l-APTMES instead of APTMES can lead to a more efficient CFZ adsorption. The addition of OLA as a NP stabilizing agent in ethanol favors CFZ adsorption. When a lipid bilayer is added on top of the functionalized surface, we observe that its leaflets exhibit an asymmetric lipid distribution, due to their interactions with the ligand layer. Regarding CFZ, most of the molecules are most favorably adsorbed in the region delimited by the ligand layer and the lower lipid bilayer leaflet. In the next steps, we aim to investigate the possible mechanisms and the energetics of drug release.

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RATIONAL DESIGN OF DRUGS TO INHIBIT AN 'IRRATIONAL' PROTEIN TARGET

Bruno Rizzuti[1,2]

[1]National Research Council - Institute of Nanotechnology (CNR-NANOTEC), SS Rende (CS), Department of Physics, University of Calabria, 87036 Rende, Italy
[2]Institute for Biocomputation and Physics of Complex Systems (BIFI), University of Zaragoza, 50018 Zaragoza, Spain
bruno.rizzuti@cnr.it

NUPR1 is an intrinsically disordered protein involved in the progression of several tumors. It is completely unfolded, either in isolation or in complex with any molecular partner, and spans a highly dynamic ensemble of conformations. Traditional biophysical techniques struggle to define the behaviour of such an abnormal (or 'irrational') protein target, and they can be aided by computational methods in the quest for designing drugs to inhibit its function.

Initial bioinformatic analysis of the NUPR1 sequence helped to identify some of the key physical properties driving ligand binding and to screen a series of hits, including several drugs previously approved for human use, such as the antipsychotic trifluoperazine.[1] This repurposed compound was later optimized through a ligand-based drug design process, resulting in the patenting of a class of new anticancer molecules.[2] The best-in-class of these was considered the lead compound and, more recently, a further computational modeling endeavor was carried out in an attempt to improve its binding affinity.[3] In parallel, a number of alternative and completely different molecular scaffolds have been considered, and some are currently being tested to avoid the side effects of trifluoperazine that are common to its derivatives.[4] Along the way, molecular simulations helped to prove that inhibition of NUPR1 could also be achieved by using larger molecules, including helical peptides[5] (with an amphipathic charge distribution) and functionalized dendrimers[6] (either anionic, cationic, or neutral).

The reported results provide an example of how *in silico* techniques can be used to support *in vitro*, *in cellulo*, and *in vivo* experiments in a comprehensive drug discovery and development funnel. Notably, simulations were here applied to an unfolded protein lacking any well-defined binding pocket. This observation suggests that intrinsically disordered proteins, while still being considered abnormal drug targets, should no longer be deemed 'undruggable'.

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MATHEMATICAL MODELING OF KINEMATICS AND EXPERIMENTAL CONTROL OF CYTOKINE EXPRESSION FOR THE CHARACTERIZATION OF PRO- AND ANTI-INFLAMMATORY PATHWAYS IN MACROPHAGES

Jennifer Riccio,[1] Luca Presotto,[1] Liad Doniza,[2] Uri Nevo,[2] Donato Inverso,[3] and Giuseppe Chirico[1]

[1]Department of Physics, Università degli Studi di Milano-Bicocca, Piazza della Scienza 3, 20126 Milan, Italy
[2]Department of Biomedical Engineering, Tel Aviv University, Haim Levanon St. 30, Ramat Aviv, 6997801, Tel Aviv, Israel
[3]Division of Immunology, Transplantation and Infectious Diseases, Vita-Salute San Raffaele University, Via Olgettina 58, 20132 Milan, Italy
jennifer.riccio@unimib.it

Macrophages are immune cells capable of exhibiting a functional phenotype depending on the surrounding tissue environment (polarization process). Macrophage polarization can be identified by characteristic markers expressed by the cell itself and classified into a continuous spectrum of activation states with extremes typically labeled as pro-inflammatory or M1 and anti-inflammatory or M2.[1] This dynamic process has been described by several mathematical models which attempt to reproduce signaling multi-pathways at subcellular level in response to different types of stimuli.
In this scenario, the objective of this work is to quantify the immune reaction induced by the presence of biomaterials at the cellular level, with the final goals to develop a simulation platform to assist the evaluation of biomaterial reaction and, in so doing, help to reduce the number of used animals, the costs of biomaterial discovery and protocols for biocompatibility. To do that, we first consider the ODE model proposed by Maiti et al.[2] to simulate single-cell kinematics. This is based both on literature references and experimental results and includes a pro- and an anti-inflammatory module, representing TNF-α and IL-10 cytokines, respectively, through feedback regulations on NF-kB factor, under constant lipopolysaccharide (LPS) stimulation. We run the model for 12 hours providing a LPS concentration value of 1 pg/mL. Cytoplasmatic concentration of TNF-α (TNF-α *cyto*) from simulation is compared to experimental results based on fluorescence-activated cell sorting (FACS) analysis, obtained by incubating murine macrophages with the same LPS stimulus at six different time points up to 12 hours. Our study highlights that TNF-α *cyto* reaches its peak value around the 1 h simulation time, after which the percentage of cells expressing TNF-α also starts to increase. In this context, future analyses also targeting IL-10 and NF-kB, as well supernatant and mRNA concentrations by means different in-vitro experiments (ELISA and PCR, respectively), will be performed to better understand the temporal evolution of these species. In parallel, agent-based models have been evaluated, to consider interactions among pro- and anti-inflammatory mediators and quantify cell-to-cell variability. Finally, mechano-transduction will be included, so as to provide a comprehensive modeling merging biomechanics and cytokines signaling.

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IDENTIFICATION OF CRYPTIC POCKETS VIA BIASED MOLECULAR DYNAMICS

M. Rigoli[1], R. Tandiana[1], M. A. Soler[2], and W. Rocchia[1]

[1]Computational MOdelling of NanosCalE and BioPhysical SysTems-CONCEPT Lab,Istituto Italiano di Tecnologia (IIT), Via Melen-83, B Block, 16152 Genoa, Italy

[2]Dipartimento di Scienze Matematiche, Informatiche e Fisiche, Universita' di Udine, Via delle Scienze 206, 33100 Udine, Italy

Cryptic pockets consist of hidden binding sites within the three-dimensional structure of a protein that often remain inactive or inaccessible under normal physiological conditions but can become functional upon environmental modifications, ligand binding, or structural alterations. These pock- ets typically arise due to the intrinsic dynamical properties of protein structures, where conforma- tional changes may occlude or expose certain regions of the protein surface. Furthermore, they can play crucial roles in various biological processes, including molecular recognition, signal transduc- tion, and enzymatic activity regulation. The identification and characterization of hidden binding sites have gathered significant attention in structural biology and drug discovery, as they repre- sent potential targets for the design of novel therapeutics and modulators that can selectively bind and regulate protein function. Computational methods, such as Molecular Docking and Molecular Dynamics (MD) simulations, along with experimental techniques like X-ray crystallography and nuclear magnetic resonance spectroscopy, are commonly employed to elucidate the presence and properties of cryptic pockets in proteins. Among the computational techniques that have been developed to identify hidden pockets, we find Hamiltonian Replica Exchange techniques[1], and mixed-solvent MD simulations[2,3] that have been widely applied to address this complex task. In this context, we used a computational approach previously developed by our group[4], which is an enhanced sampling technique based on the fine-tuning of the system's hydration. In more detail, we employed biased MD simulations that exploit accelerated dynamics using a collective variable represented by solute hydration. Furthermore, the trajectories were analyzed with software, namely SiteFerret[5] and Fpocket[6], which aims at identifying ligandable pockets on the protein surface. In this work, we studied the SARS-CoV-2 spike protein's receptor binding domain (RBD) with the aim of discovering druggable cryptic pockets. The RBD system was simulated through enhanced sampling MD at different hydration values, and it was possible to observe the formation of a pocket that was neither present in the native state nor observed during plain MD trajectories. Our re- sults are in line with some recent findings by Zuzic and colleagues[7], who were able to observe the aforementioned cryptic binding site of SARS-CoV-2 spike protein, by combining in silico and experimental protein structure determination techniques, and with the work of Toelzer et al. that solved the 3D structure of the SARS-CoV-2 spike protein in complex with a fatty acid ligand[8].

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BIOFISICA APPLICATA

NANOGELS FROM GUANOSINE HYDROGELS: UNVEIL A NEW DRUG DELIVERY SYSTEM

Paolo Moretti, Alessia Pepe, Francesca Ripanti, Paolo Mariani

Università Politecnica delle Marche, Dipartimento di Scienze della Vita e dell'Ambiente, Ancona, Italy
paolo.moretti@univpm.it

The investigation and the development of nano - systems aimed at drug delivery and target therapy is a new frontier in the biotechnological world [1]. In this scenerio, Nanogels (NGs) have been recognized as excellent carriers to obtain these functions in a lot of biotechnological applications involving a large spectrum of disease [2]. We developed a new NG derived from the physical guanine (G)-hydrogel. Indeed, two guanine derivatives enter as gelators in the composition of G-hydrogel, guanosine (Gua) and the guanosine 5'- monophosphate (GMP). The hydrogel is capable to swell up to a water content of 99% (v/v). The hydrophilicity of GMP allows solubilizing the hydrophobic Gua, forming G-quartets by non-covalent Hoogsteen bonds. The stacking of this G-quartets guides the formation of a columnar helicoidal structures (G-quadruplex) that constitute the strands of the G-hydrogel 3D network. [3,4,5]. We developed a protocol to synthesize G-NGs in KCl. We proved that is possible to modulate the characteristics of the G-NGs by changing the Gua/GMP molar ratios (1:1, 1:2, 1:4). We also tested the effects of different polymers (Pei, Poloxamer and PolyLysine, at different concentrations) on the G-NG stability and demonstrated that we are able to tune nanoparticles size in a wide spectrum of dimensions. G-NGs structural properties have been investigated by Atomic Force Microscopy and Dynamic Light Scattering to understand morphological characteristics and stability as a function of temperature and ageing time. We performed Small Angle X-ray Scattering to obtain information on the inner structure of the G-NGs and also on the interactions with the particles in the different conditions. Finally, the confocal laser scanning microscopy showed the shape of G-NGs thanks to fluorescence probe (e.g. Thioflavin T) loaded in the nanoparticles. These preliminary results on stability and characterization on G-NGs clear the way to the possibility to use a new supramolecular, self-assembling system in the field of nano-biotech for the development of drug delivery systems for specific molecules.

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PHASOR-FLIM ANALYSIS OF CELLULOSE AGING PROCESS

Vittorio Ferrara[1], Valeria Vetri[1], Bruno Pignataro[1], Delia Francesca Chillura Martino[2], Giuseppe Sancataldo[1]

[1]Department of Physics and Chemistry - Emilio Segrè, University of Palermo, Viale delle Scienze, Palermo 90128, Italy.
[2]Department of Biological, Chemical and Pharmaceutical Sciences and Technologies (STEBICEF), University of Palermo, Viale delle Scienze, 90128 Palermo, Italy.
giuseppe.sancataldo@unipa.it

Cellulose is a natural biomacromolecule comprising the primary structural component of plant cell walls. It forms long, linear chains, providing strength and rigidity to plant cells. Its remarkable properties include high tensile strength, insolubility in water, and biodegradability. Beyond its structural role, cellulose serves as a vital renewable resource in industries such as paper, textiles, and biofuels and its molecular arrangement regulates its properties (e.g. mechanical, optical, chemical). In this scenario, oxidation and hydrolysis are recognized as the most prominent degradation patterns occurring during cellulose aging under common conditions of storage.
This study focuses on the Phasor FLIM investigation of the aging process of pure cellulose, in paper form, at varying controlled oxidation degrees. Spectral and lifetime fluorescence microscopy, along with established characterization methods, was employed to elucidate structural changes during the aging process. Carbotrace 680, a recently developed fluorescent dye with a selective affinity for glucans, was utilized to stain the paper samples. FLIM combined with a phasor approach, was employed to track and localize molecular-level changes induced by the oxidation process. The results indicate that the oxidizing treatment immediately increases cellulose molecular crystallinity, resulting in a shortening of the Carbotrace 680 fluorescence lifetime. Subsequently, by increasing the degree of oxidation a shift of the Carbotrace 680 lifetime towards longer lifetime value was observed. Notably, by the use of the microscopy approach, fine spatial information has been acquired allowing to map changes occurring upon oxidation at single fiber level. This approach indeed provides a means to visualize the spatial heterogeneity of the oxidation process, allowing for the localization of submicron-scale modifications in both the internal and external parts of individual fibers.

MONITORING BY OPTICAL BIOSENSING

Annalisa D'Arco,[1] Tiziana Mancini[1], Rosanna Mosetti[1,2], Salvatore Macis[1], Giancarlo Della Ventura[3], Augusto Marcelli[4], Cleofe Palocci[5], Laura Chronopoulou[5], Pasquale D'Angelo[6], Giuseppe Tarabella[6], Vardan Galstyan[6,7] and Stefano Lupi[1]

1 Department of Physics, University of Rome La Sapienza, P.le A. Moro 2, 00185 Rome, Italy
2 Department of Basic and Applied Sciences for Engineering, University of Rome La Sapienza, Via Scarpa 16, 00161 Rome, Italy
3 Department of Science, University Rome Tre, Largo San Leonardo Murialdo 1, 00146 Rome, Italy
4 Laboratori Nazionali Frascati, National Institute for Nuclear Physics (INFN-LNF), Via E. Fermi 54, 00044 Frascati, Italy
5 Department of Chemistry, University of Rome La Sapienza, P.le A. Moro 2, 00185 RM, Italy
6 Institute of Materials for Electronics and Magnetism, National Research Council (IMEM-CNR), Parco Area delle Scienze, 37/A - 43124 Parma (PR), Italy
7 Department of Engineering "Enzo Ferrari", University of Modena and Reggio Emilia, Via Vivarelli 10, 41125 Modena, Italy
annalisa.darco@uniroma1.it

Portable and real-time devices for biosensing have various application fields, including air-quality monitoring for occupational health, safety, and security. In the scenario of air-quality, there is increasing need for technological advancements in the field of sensors for atmospheric pollutants, with the aim of monitoring with the highest sensitivity and accuracy the type and the amount of chemical and biological pollutants. In particular, as the recent SARS-CoV-2 crisis [1] clearly showed, monitoring the dispersion of pathogens is a challenging but crucial and necessary issue to prevent the spread of a pandemic and to guarantee health safety, in particular in close public indoor spaces and working sites [2].
Motivated by the request to monitor the airborne pathogens, we illustrate the steps toward developing infrared TIO_2 nanostructured arrays. Herein, we fabricated TiO_2 nanotubes (NTs) by electrochemical anodization method and investigated the prepared samples as a platform for label-free optical monitoring of biomolecules, such as SARS-CoV-2 spike glycoproteins. To improve the selectivity of sensors based on TiO_2 NTs, we chemically modified their surface [3] to bind the spike glycoprotein. The process of biofunctionalization was monitored and validated by using optical methods, including Infrared (IR) spectroscopy and UV-Vis spectroscopy [4]. Our experimental findings show that TiO_2 NTs array can be used as a versatile platform for the detection of biochemical molecules that are interrogable with optical methods. Moreover, IR spectroscopy has proven to be an effective tool able to the recognizing and monitoring pathogens. Our work paves the way for the analysis of various other nanostructured platforms and optimization of the biofunctionalization process to develop an effective optical biosensor for airborne pathogens.

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LIPOPROTEIN QUALITY ANALYSIS USING RAMAN SPECTROSCOPY IN A CLINICAL SETTING

Arianna Bonizzi[1,2], Flavia Magri[2,3], Serena Mazzucchelli[1], Andrea Rizzi[4], Fabio Corsi[1,2], Roberta Cazzola[1], Carlo Morasso[2]

1. Department of Biomedical and Clinica l Sciences, Università degli Studi di Milano, Via G.B. Grassi 74, 20157 Milan, Italy
2. Istituti Clinici Scientifici Maugeri IRCCS, Via Maugeri 4, 27100 Pavia, Italy
3. Department of Internal medicine and Medical Therapy, Università degli Studi di Pavia; C.so Strada Nuova, 27100 Pavia, Italy
4. S.C. Chirurgia Generale Tradate, Ospedale Galmarini di Tradate, ASST dei Sette Laghi, 21049 Tradate, Italy arianna.bonizzi@unimi.it

Lipoproteins (LPs) play a crucial role in lipid transport within the body. Routine measurement of cholesterol (CL) levels transported in the LPs is one of the main clinical approaches used to assess the risk of cardiovascular events (CVD). However, traditional CL assessment techniques do not fully capture the *quality* of LPs, related to their biochemical composition and functional complexity, which have been found to be altered in various pathological conditions. Nonetheless, the quality analysis of the LPs lacks a generally accepted gold standard method. In this context, Raman Spectroscopy (RS) represents a promising tool to obtain relevant information on LPs *quality* rapidly and efficiently [1,2]. The ability of RS to extract information on the chemical fingerprint of LPs was tested in a clinical study aimed to profile the main classes of LPs extracted by ultracentrifugation in a discontinuous KBr density gradient from human plasma of obese patients [HC, n= 39] with BMI ≥ 30 kg/m^2 compared to a control group formed by healthy volunteers free from any significant disease [HC, n= 26]. Raman analysis revealed significant differences in the biochemical composition and oxidative states of LPs from the two groups. Ob's LDL exhibited reduced carotenoids (1525 cm-1) and unsaturated lipid levels (960 cm-1, 1271 cm-1, 1656 cm-1), while Ob's HDL showed variations in cholesterol (700 cm-1), phospholipids (717 cm-1), and lipid/protein ratio (2851/2930 cm-1) compared to HC, suggesting potential implications for cardiovascular health.
Overall, these results demonstrate the potential of RS for fast and comprehensive assessment of LPs *quality* in clinical settings and pave the road toward a more comprehensive understanding of the role of LPs in metabolic disorders.

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G-HYDROGEL: FROM TRANSPORT PROPERTIES TO AN INNOVATIVE BIO-INK FOR 3D CELLULAR SCAFFOLD

Alessia Pepe, Paolo Moretti, Francesca Ripanti, Paolo Mariani

Università Politecnica delle Marche, Dipartimento di Scienze della Vita e dell'Ambiente (DiSVA), Ancona, Italy

It is well known that a mixture in water of Guanosine 5'-monophosphate (GMP) and Guanosine (Gua) at the correct composition leads to the formation of a supramolecular physical gel, able to trap very large amount of water (up to 99% v/v). Indeed, GMP and Gua in solution form G-quartets, which stack each other giving rise to G-quadruplex helicoidal strands[1] . While GMP molecules have negative charges and then provide G-quadruplex repulsions, uncharged Gua molecules determine van der Waals interactions and an increased flexibility of G-quadruplex portions (knots)[2,3]. Therefore, the correct balance of GMP and Gua induces the formation of stable hydrogels, whose structural and mechanical properties, including viscosity, can be easily modulated. In this study, we initially pointed out some new features of G-hydrogel, such as transport properties, and their dependence on composition. Fluorescent Recovery After Photobleaching (FRAP) experiments were performed on G-hydrogels prepared at different Gua/GMP composition and water content, in order to derive diffusivity properties for a series of fluorescent dyes of different characteristics. DAPI, ThT and rhodamine B were taken as small hydrophobic, weakly hydrophobic and hydrophilic molecules, respectively, while FITC-dextrans at various molecular weights were considered to represent large hydrophilic systems. FRAP experiments provided the diffusion constants for all the considered molecules, but a low diffusivity rate for ThT and DAPI was detected, because of strong interactions with G-quadruplexes. As important support, UV-vis spectroscopy, Small-Angle and Wide-Angle X-ray Scattering and Atomic Force Microscopy experiments were performed to assess eventual changes in the G-hydrogel structural properties[4] . Because of self-formation, self-healing, good injectability, complete transparency, high thermal and thermodynamics stability, G-hydrogels appear very suitable for several biotech and biomedical applications. Here, the usage of this hydrogel as a novel bio-ink ideal for 3D printing of cellular scaffolds, as well as the influence that the hydrogel mechanical and structural properties have on cell behaviour and the role of mechanical stimulation in cell-seeded hydrogels, will be described.

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SPECTROSCOPIC ANALYSIS OF VISIBLE BLUE LIGHT-ACTIVATED PHOTOCATALYSIS ON BACTERIA-LIKE TARGETS

Giorgia Puleo, [1,2] Vittorio Ferrara, [3] Lucrezia Caselli,[4] Giuseppe Sancataldo, [3] Mariano Licciardi, [1] Vito Foderà, [2] Valeria Vetri [3]

1 Department of Biological, Chemical and Pharmaceutical Sciences and Technologies (STEBICEF), University of Palermo, Viale delle Scienze Bld.18, Palermo, 90128, Italy
2 Department of Pharmacy, University of Copenhagen, Universitetsparken 2, Copenhagen, 2100, Denmark
3 Department of Physics and Chemistry, University of Palermo, Viale delle Scienze Bld. 18, Palermo, 90128, Italy
4 Physical Chemistry 1, Lund University, Lund S-221 00, Sweden
* giorgia.puleo01@unipa.it

Investigating novel and highly effective photocatalytic agents for antibacterial Photodynamic Therapy (aPDT) presents a novel strategy against the escalating challenge of multidrug-resistant bacterial infections. aPDT exploits the production of Reactive Oxygen Species (ROS) upon exposure of photosensitizers to light. Here we present a study on the synergistic effect of Nitrogen-doped Titanium Dioxide (N-TiO2), as photodynamic agent, in combination with gold nanorods (AuNRs) as enhancing components. Nitrogen doping was adopted during the synthesis to modify the bandgap of TiO2 and make it activatable by visible light (1). The photocatalytic efficacy of N-TiO2, both alone and in combination with AuNRs, was assessed using optical spectroscopy using on Methyl Orange as a standard dye commonly used to this purpose. Interestingly, it was found that effective photodegradation of the dye under blue light was enhanced in the presence of AuNRs. This can be attributed to the ability of AuNRs to stabilize charge carriers on photocatalytic surfaces, thereby enhancing photocatalytic performances (2). With the idea of evaluating the ability of these nanostructure composites of photodegrading molecular targets present in bacteria, emphasis was placed on the evaluation of damage to the DNA helix and oxidation of lipid membranes. A through spectroscopic analysis revealed significant alterations in DNA structures, with critical modifications emphasized in the presence of AuNRs. These modifications were caused by conformational changes of the double helix, as a result of nucleotide bases oxidation. Consistent with these results, an oxidation assay on lipid vesicles demonstrated increased oxidation rates in the presence of AuNRs, suggesting that the photocatalytic process may impact cell membranes, damaging them.

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A STRUCTURAL INVESTIGATION OF AN INNOVATIVE NANOCONSTRUCTDESIGNED FOR THERANOSTIC AGAINST MULTIPLE MYELOMA

Alice Piccinini [1], Lorenzo Dal Fabbro[1], Nicolo` Percivalle[3], Sally Helmy[1,2], Valentina Cauda[3], Paola Brocca[1], Valeria Rondelli[1]

1 Universita` Statale di Milano, Department of Medical Biotechnology and Translational Medicine
2 Biophysics Group, Physics Department, Faculty of Science, Ain Shams University, Cairo, Egypt
3 Politecnico di Torino, Department of Applied Science and Technology
alice.piccinini@unimi.it

Multiple myeloma (MM) is the second most abundant hematologic malignancy of the plasma cells in adults, often treated with pharmacological therapy and autologous stem cell transplant. Nowadays, an innovative approach to cancer treatment is represented by nanotheranostics [1], which exploits biocompatible and functionalized nanoparticles (NPs) allowing the diagnosis of cancerogenic lesions in their earliest stages, as well as targeted cancer treatment. Our project aims specifically to deliver a stimuli-responsive NP characterized by a metal core[2] carrying an anticancer drug, embedded in a shell of mixed synthetic and natural lipids. We conducted a biophysical study to comprehend the NP's behaviour and ruling NP's structure and stability. By Small-Angle X-ray scattering (SAXS) techniques, we could determine the NP's structure and we have been able to evaluate the effect of each stage of NP-core's functionalization treatments on overall structure. In particular, the next step aimed to envelop the metal core of the NP with the lipidic shell, has been investigated with a variety of experimental techniques, mainly neutron reflectometry, small and wide angle X ray scattering and differential scanning calorimetry. We have been able to evaluate that the structure and thermal behaviour of the lipid coating is not dramatically altered after interaction with the NP-core. Lastly, our attention was focused on the interaction of the NP with model membranes, mimicking the contact with the target cells. We employed membrane models of different complexity and we demonstrated a non-randomly fusogenic behaviour of the NP, a crucial characteristic for the efficient delivery of the drug. Our work clarifies the structure and behaviour of a rather complex drug delivery system and its behaviour in the presence of target membranes and, in addition to being a beneficial investigation for the MM treatment, is an example of how biophysical techniques can be employed to optimise the design of drug delivery systems.

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BIOFISICA CELLULARE E DEI TESSUTI

BRILLOUIN SPECTROSCOPY FOR THE MICROMECHANICAL CHARACTERIZATION OF CELLS AND TISSUES

Passeri, A.A.[1], Bonacci, F. [1], Fioretto, D.[1] , Mattarelli, M.[1], Caponi, S.[2]

[1] Department of Physics e Geology, University of Perugia, I-06100 Perugia, Italy
[2] Istituto Officina dei Materiali, National Research Council of Italy (CNR-IOM), Unit of Perugia, 06123 Perugia, Italy
silvia.caponi@cnr.it

The mechanical interactions are fundamental elements that drive the behaviour of living matter. In the last decade, Brillouin microscopy (BM) has shown great potentiality as a new optical elastography method for the micromechanical characterization of biomatter [1, 2]. Based on the interaction between light and matter, this spectroscopic technique offers the opportunity to obtain elastography maps in contactless and non-invasive way. Our team developed a correlative Brillouin and Raman microscope[3,4] which offers the unique opportunity to correlate the local mechanical and chemical properties of the materials with sub-cellular spatial resolution. This instrument demonstrated its performance in a wide range of applications spanning from basic research to bio-medical science. The capability of this investigation method is reviewed in this talk presenting relevant results obtained in the framework of cell mechanobiology and tissue histopathology.

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HIGH RESOLUTION FTIR IMAGING OF ORAL TONGUE SQUAMOUS CELL CARCINOMA: NEW INSIGHTS ON TUMOR STAGING

Valentina Notarstefano,[1] Alessia Belloni,[1] Chiara Santoni,[1] Paolo Mariani,[1] Elisabetta Giorgini[1]

[1]Dipartimento di Scienze della Vita e dell'Ambiente, Università Politecnica delle Marche, Ancona
v.notarstefano@univpm.it

Oral Tongue Squamous Cell Carcinoma (OTSCC) is the most common malignancy of the head and neck region.[1] Pathological staging is primarily based on the morphology of the tumor parenchyma. Recently, the peritumoral region, the tissue surrounding the tumor mass, has gained interest as it plays an essential role in tumor growth and metastasis. Fourier Transform InfraRed Imaging (FTIRI) represents a versatile technique able to provide a high resolution morpho-chemical analysis of biological samples.[2] Hence, to improve OTSCC early diagnosis, tumor biopsies were analyzed by FTIRI to identify reliable spectral biomarkers of the tumor region at different stages, together with the macromolecular alterations associated with these biomarkers in the peritumoral areas.

The intra-, peri- and extratumoral regions of N.35 OTSCC biopsies were investigated and the IR images (164×164μm size, 4096 pixels/spectra, 2.56×2.56μm spatial resolution) collected in transmission mode in the 4000-900 cm^{-1} range.

The hyperspectral imaging analysis evidenced a profound difference of the macromolecular composition between intra- and peri-/extra-tumoral regions, with the first being mainly composed of phosphates (nuclei) and the two latter of collagen: this confirmed the cellulated nature of the tumor mass, rich in nucleic acids and poor in collagen. Moreover, the peri-tumoral areas of advanced tumor stages clearly displayed a decrease in the relative amount of collagen, together with a reduction of alpha and triple helices and an increase of random structures, suggesting an involvement of this border region in the tumor growth.

Based on these results, the spectral features related to collagen structure of the peri-/extra-tumoral regions resulted to be reliable and promising markers to improve the discrimination between early and advanced tumor stages, paving the way to implemented and more efficient pathological staging approaches.

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SIGNAL TRANSMISSION AT VESTIBULAR TYPE I HAIR CELLS

Paolo Spaiardi,[1,2] Roberta Giunta,[1] Giorgio Rispoli,[3] Sergio Masetto[1†] and Stuart L. Johnson[4†]

[1]Department of Brain and Behavioural Sciences, University of Pavia, Pavia, Italy.
[2]Department of Biology and Biotechnology, University of Pavia, Pavia, Italy.
[3]Department of Neuroscience and Rehabilitation, University of Ferrara, Ferrara, Italy.
[4]Sheffield Neuroscience Institute, University of Sheffield, Sheffield, United Kingdom
rsg@unife.it

Head movements are detected and signalled to primary sensory neurons by vestibular type-I and type-II hair cells located in the inner ear. Signal transmission involves glutamate exocytosis from hair cells, which is triggered by Ca^{2+} inflow[1,2] through voltage-gated $Ca_V1.3$ Ca^{2+} channels. In a previous study[3] in mice, by measuring the change in cell membrane capacitance (ΔCm) elicited by a depolarization we reported a sustained exocytosis in both hair cell types, which was significantly smaller in type-I than in type-II hair cells. However, type-I hair cells also showed a large transient ΔCm. At difference from the sustained ΔCm, the transient ΔCm was still present in *$Ca_V1.3^{-/-}$* mouse type-I hair cells. In the present study we have further investigated the nature of the transient ΔCm in mouse mature vestibular type-I hair cells. We found that the transient ΔCm was not affected by 10 mM intracellular EGTA, which conversely impaired the sustained exocytosis elicited by depolarizing voltage steps in both type-I and type-II hair cells. Moreover, the amplitude of the transient ΔCm correlated with the degree of activation of the low-voltage activated outward rectifying K^+ conductance, $G_{K,L}$, which is expressed by type-I, but not type-II hair cells[4]. Finally, the sign and the kinetics of the transient ΔCm changed according to voltage steps activating or deactivating $G_{K,L}$. The above results are consistent with the transient ΔCm arising from the mobilization of charges during gating of K,L channels. The large size of the transient ΔCm is consistent with a high resistance of the calyceal synaptic cleft since its amplitude, but not that of $G_{K,L}$, was reduced in *$Caspr^{-/-}$* mice, which show a significantly larger synaptic cleft compared to WT mice.

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PROGRESSIVE ALTERATION OF MURINE BLADDER ELASTICITY IN ACTINIC CYSTITIS DETECTED BY BRILLOUIN MICROSCOPY

Claudia Testi[1], Laura Martinez-Vidal[2,3], Massimo Alfano[2,3], Emanuele Pontecorvo[4], Giancarlo Ruocco[1,5]

1 Center for Life Nano- and Neuro-Science, Istituto Italiano di Tecnologia, Viale Regina Elena 291, 00161, Roma, Italy
2 Division of Experimental Oncology/Unit of Urology, IRCCS Ospedale San Raffaele, 20132, Milan, Italy
3Università Vita-Salute San Raffaele, Via Olgettina, 60, 20132, Milan, Italy
4CrestOptics S.p.A., Via Di Torre Rossa, 66, 00165, Roma, Italy
5Dipartimento Di Fisica, Universitá Di Roma "La Sapienza", Piazzale Aldo Moro, 5, 00185, Roma, Italy
Claudia.testi@iit.it

The biological consequences of altered tissue mechanics greatly affect organs homeostasis and functions. In vivo, the local microenvironment within tissues continuously exerts mechanical forces (i.e., shear, compressive, extensional forces) on cells, triggering the activation of a cascade of biomechanical pathways critical for morphogenesis, homeostasis and development[1].

Bladder mechanical properties are critical for proper organ function and tissue homeostasis: alteration of its elasticity is thus linked to disease onset and progression[2]. This study aims to characterize the tissue elasticity of the murine bladder wall in healthy conditions and in actinic cystitis, a state characterized by tissue fibrosis that is a frequent complication of radiation therapy. Here, we exploit Brillouin microscopy, an emerging technique in the mechanobiology field that allows mapping tissue mechanics at the microscale, in non-contact and label-free mode[3].

We show that Brillouin imaging of murine bladder tissues is able to recognize different anatomical components of the bladder wall, confirmed by histopathological analysis, showing different tissue mechanical properties of the physiological bladder, as well as a significant alteration in the presence of tissue fibrosis. Our results point out the potential use of Brillouin imaging on clinically relevant samples as a complementary technique to histopathological analysis, deciphering complex mechanical alteration of each tissue layer of an organ that strongly relies on mechanical properties to perform its function[4].

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STRUCTURAL AND FUNCTIONAL REPROGRAMMING OF ADIPCOCYTES DURING MATURATION AND HYPERTROPHY

Lama Zeaiter[1,2], Francesca Baldini[2], Hawraa Zbeeb[1], Lisa Cuneo[2,3], Paolo Bianchini[2], Piero Portincasa[4], Alberto Diaspro[2,3], and Laura Vergani[1]

[1]DISTAV, Department for the Earth, Environment and Life Sciences, University of Genoa, Genoa, Italy
[2]Nanoscopy, Istituto Italiano Tecnologia, Genoa, Italy
[3]DIFILAB Department of Physics, University of Genoa, Genoa, Italy
[4]Clinica Medica "A. Murri", Department of Biomedical Sciences and Human Oncology, University of Bari, Bari, Italy

The escalating prevalence of obesity worldwide represents a serious public health challenge. This "obesity epidemic" requires a better understanding of the molecular and physiological pathways addressing healthy adipocytes towards hypertrophy, the main responsible of adipose tissue expansion. Hypertrophic adipocytes develop an inflammatory phenotype with altered secretion of adipokines, fatty acids and soluble factors which impact on many organs and contribute to hepatic steatosis and insulin resistance. We think obesity as a physiological reprogramming of adipocyte function mediated by nuclear architecture and chromatin epigenetics remodeling. As in vitro model of adipogenesis and hypertrophy the 3T3-L1 cell line was employed. Confocal and super-resolution stimulation emission depletion (STED) microscopy combined with ELISA assays, real time PCR and high-resolution respirometry allowed us to explore nuclear architecture, chromatin distribution, epigenetic rearrangements, gene expression profile, and mitochondrial respiration. The hypertrophic phenotype was characterized by larger fat depots, increased expression of PPARγ adiponectin and metallothioneins, reduced IL-1a release. At mitochondrial level, impaired oxygen consumption was measured in hypertrophic adipocytes. The confocal microscopy revealed larger volume and less elongated shape of the nuclei during adipogenesis and hypertrophy with a trend of reduced chromatin compaction. The epigenome remodeling in hypertrophic adipocytes showed an increased H3K9 acetylation and a global DNA hypomethylation. Moreover, by the conditioned medium method we assessed how hypertrophic adipocytes might affect co-cultured hepatocytes leading to steatosis and metabolic dysfunction. The present findings demonstrate a structural and functional comprehensive remodeling of adipocyte nuclei underlining their inflammatory phenotype which also influence the metabolic status of liver cells.

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A BIOPHYSICAL STUDY OF THE DYNAMIC PROPERTIES OF GLUCAGON GRANULES IN A CELLS BY IMSD AND SPT APPROACHES

S. Ghignoli, V. De. Lorenzi, G. Ferri, S. Luin, F. Cardarelli

Insulin and glucagon are the two essential hormones for maintaining proper blood glucose homeostasis, which is disrupted in Diabetes. A constantly growing research interest has been focused on the study of the subcellular structures involved in hormone secretion, namely insulin- and glucagon-containing granules, and on the mechanisms regulating their behaviour. Yet, while several successful attempts were reported describing the dynamic properties of insulin granules, little is known about their counterparts in α cells, the glucagon-containing granules. To fill this gap, we used αTC1 clone 9 cells as a model of α cells, and ZIGIR as a fluorescent Zinc chelator for granule labelling. We started by using spatiotemporal fluorescence correlation spectroscopy in the form of imaging-derived mean square displacement (iMSD) analysis. This afforded quantitative information on the average dynamical and structural properties of glucagon granules having insulin granules as benchmark. Interestingly, the iMSD sensitivity to average granule size allowed us to confirm that glucagon granules are smaller than insulin ones (∼1.4 folds, further validated by STORM imaging). To investigate possible heterogeneities in granule dynamic properties, we moved from correlation spectroscopy to single particle tracking (SPT). We developed a MATLAB script to localize and track single granules with high spatial resolution. This enabled us to classify the glucagon granules, based on their dynamic properties, as 'blocked' (i.e., trajectories corresponding to immobile granules), 'diffusive' (i.e., trajectories corresponding to slowly moving granules in a defined region of the cell), or 'drifted' (i.e., trajectories corresponding to fast moving granules associated to directional transport). In cell-culturing control conditions, results show this average distribution: 32.9 ± 9.3% blocked, 59.6 ± 9.3% diffusive, and 7.4 ± 3.2% drifted. This benchmarking provided us with a foundation for investigating selected experimental conditions of interest, such as the glucagon- granule relationship with the cytoskeleton. To better understand this relationship, we employed Nocodazole (10 μM) for microtubules depolymerization and LatrunculiB (10 μM) for actin filaments disruption. Moreover, we decided to perform the same stimuli on the β cells to have a control condition of the better-known behaviour of insulin granules. Interestingly glucagon granules and insulin granules behave differently specifically after actin filaments disruption. Glucagon granules seems to be less prone to perform active transport (no differences in the drifted motion), with respectto insulin granules. Noteworhtly, for glucagon granules the active transport motility (drifted motion) was decreased by Nocodazole and increased after hypoglycaemia stimuli (1mM glucose condition). These results confirm the clear link between glucagon-granule motion, cytoskeleton structures and secretion stimuli, obtaining a first insight of the intracellular behaviour of these subcellular components. The information collected might now serve to support future investigations on glucagon granules in physiology and disease. [This work has received funding from the European Research Council (ERC) under the European Union's Horizon 2020 research and innovation programme (grant agreement No 866127, project CAPTUR3D).]

PHYSICALLY INSPIRED SCATTERING CORRECTION FOR NON-LINEAR EXCITATION IMAGING OF TISSUES.

Davide Panzeri, Giuseppe Chirico, Mario Marini, Luca Presotto, Laura Sironi, Margaux Bouzin, Laura D'Alfonso, Maddalena Collini.

Dipartimento di Fisica, Università di Milano-Bicocca, piazza della scienza 3, 20126, Milano (I).

Nonlinear excitation in the near-infrared (NIR) greatly improves in vivo optical imaging by reducing tissue scattering,1 it offers intrinsic optical sectioning and it is inversely proportional to the duty cycle. However, even in the NIR, tissue scattering spreads the electric field in both space and time, resulting in only a small fraction of the injected energy reaching the target object.2 To effectively deliver light energy through a turbid medium like a biological tissue, the effect of the scattering on the propagating waves must be corrected, for example by means of a time reversal approach.3–6 However, non-isoplanatism of the scattered field collected through an optical scanning microscope is the major practical limitation to these approaches. For a 100x100 μm field of view (FOV) and an isoplanatic patch7 $\simeq 10\ \mu m$, we would need to compute about 100 different corrections phases per FOV.8 There is therefore a clear need for an optimized approach to the correction of the tissue induced scattering that minimize the correction time and increase the effective size of the correction patch on the sample. We set here the workflow of a project on the development of methods assisted by Physically Inspired neural networks (PINN) for the correction of the effect of tissue scattering on non-linear excitation microscopy, capable to partially overcome non-isoplanatism and to increase the speed of the correction loop. We model the effect of tissue scattering on the field propagating a distance d in the sample ($100 \geq d \geq 10\ \mu m$). The scattering medium is simulated by a stack of N_L layers of thickness Δ in each of which a set of N_s scatterers are embedded, spanning the stretch from z_{in} to $z_{fin} = z_{in} + N_L\Delta$ along the optical axis. The field $U(\rho_n, z_n)$ on the plane Π_n, at the position $z_n = z_{in} + n\Delta$, is computed as the sum of $U(\rho_n, z_{n-1})$ plus the contribution of the N_s random scatterers, each endowed with their own complex scattering amplitude.9 The effect of the tissue scattering is then corrected by a holographic phase shift method 3,10 with 3 dephasing angles. We tested the algorithm for the propagation of the electric field across the tissue over a field of view of $32\mu m\ x\ 32\ \mu m$, which is about 4 times the Gaussian beam waist at the initial plane Π_{in} at z_{in} = -80 μm with respect to objective focal plane. To give here an example, for $N_s = 40$ we find that the beam waist increases with the change of the refractive index of the scatterer with respect to the medium: $w_0(\delta n = 0.06) = 3.4 \pm 0.2\ \mu m, w_0(\delta n = 0.12) = 4.25 \pm 0.1\ \mu m$ and $w_0(\delta n = 0.24) = 5.1 \pm 0.1\ \mu m$. Studies as a function of the density, the size and the inhomogeneity of the tissue will be discussed. The scattering correction to the field is evaluated on the fourth power of the amplitude (two-photon excitation), employing 32 modes on the spatial light modulator (SLM). The optimization efficiency after 10 iterations on the modes as derived from the amplitude of the signal at the sample plane, is about 5 fold for $\delta n = 0.04$ and about 80 fold for $\delta n = 0.1$. We discuss then the possibility to sample different and adjacent volumes of the simulated tissue starting from a first correction matrix computed on a nearby volume and estimate the iso-planatic patch size. We then discuss the possibility to use the SLM to implement randomly structured illumination that samples the tissue over a few Airy disks by means of a pixelated array, providing a fingerprint of the scattering point spread function. Two interconnected neural networks will be fed by these fingerprints to retrieve both the optimal SLM phase and a physical description of the tissue scattering sources. We will leverage on physical models of the tissue scattering developed above and the partial superposition of excitation light cones in the tissue to propagate the tissue scattering correction between closely spaced non-isoplanatic patches.

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BIOFISICA ALLA NANOSCALA

ADVANCED OPTICAL NANOSCOPY TO HIGHLIGHT THE EFFECTS ON CHROMATIN ORGANIZATION INDUCED BY THE INTERACTION BETWEEN CELLULAR SYSTEMS AND 3D-PRINTED INTELLIGENT SCAFFOLDS

Blanca Bruschi[1], Victoire Neguembor[2], Raffaella Magrassi[3], Luana Persano[5], Andrea Camposeo[5], Francesca Cella Zanacchi[1,4], Dario Pisignano[1,5]

[1]Physics Department 'E. Fermi', University of Pisa, 56127 Pisa, Italy
[2]CRG, Centre for Genomic Regulation, 08003 Barcelona, Spain
[3]CNR-IBF, Institute of Biophysics CNR, 16149 Genova, Italy
[4]Nanoscopy and NIC@IIT, Istituto Italiano di Tecnologia, 16152 Genoa, Italy
[5]NEST, Istituto Nanoscienze-CNR, 56126 Pisa, Italy
b.bruschi@studenti.unipi.it

Cell growth and differentiation are known to be influenced by several biochemical factors. In addition, recent studies show that also environment has a strong impact on cell function[1]. Material properties such as substrate stiffness and surface topology may modulate the biophysical signals directing stem cell differentiation. Substrate stiffness has also been shown to modulate chromatin organisation at the nanoscale[2]. Although in the last few years several efforts were focused on the characterization of the nanoscale chromatin organization in stem cells[3], the precise mechanisms by which biophysical cues regulate spatial nano-scale organization remain unclear. The aim of this study is to use super-resolution microscopy, namely Stochastic Optical Reconstruction Microscopy (STORM), to characterise the chromatin organization following scaffold-cells interaction. Firstly, we characterized the chromatin of mouse embryonic stem cells (mESC) and of neuronal precursor cell (mNPC) growth in a traditional 2D culture. We quantified the difference between chromatin organization of mESC and mNPC using STORM coupled with Cluster Analysis and other microscopy-based approaches like Edge Analysis[4]. Subsequently, we used a Bioplotter to fabricate 3D-printed scaffolds made of polycaprolactone (PCL), a biocompatible material that can influence cell behavior[5]. We fabricated and characterised single uniform layer scaffolds, as well as 3D scaffolds with precise geometry. Cultures on 2D and 3D scaffolds characterized in their differentiation and chromatin organization complete the work. This provides novel knowledge in the relationship between biophysical cues and chromatin organization, providing insights into how microscale environmental changes affect cell behaviour.

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MULTIMODAL 2PEF SHG IMAGE SCANNING AND STED MICROSCOPY

Paolo Bianchini[1,3], Gaytri Arya[1], Marco Scotto D'Abbusco[1], Simonluca Piazza[2], Marco Castello[2], Alberto Diaspro[1,3]

[1]Nanoscopy and NIC@IIT, Istituto Italiano di Tecnoologia, Genoa, Italy
[2]R&D dept., Genoa Instruments, Genoa, Italy
[3]DIFILAB, Dipartimento di Fisica, Università degli Studi di Genova, Genoa, Italy
paolo.bianchini@iit.it

The use of two-photon excitation fluorescence microscopy (2PEM) has greatly advanced biological imaging, offering advantages in deeper tissue penetration, reduced photodamage, and improved three-dimensional resolution. In this study, we explore the implementation of a cutting-edge ultrafast laser system with a pulse duration of 15 fs for enhanced two-photon excitation in fluorescence microscopy. Our setup includes a single-photon avalanche diode (SPAD) array for image scanning microscopy (ISM) and a 775 nm pulsed laser for Stimulated Emission Depletion (STED) microscopy. The ultrashort excitation pulse duration allows for the excitation of fluorophores with higher peak powers and wider bandwidth, resulting in improved signal-to-noise ratios and image clarity[1,2,3]. Through ISM, we utilise a 7x7 SPAD array (Genoa Instruments s.r.l., Genoa, Italy) for an open pinhole descanned configuration, which is advantageous for 2PEM imaging. This technique has shown potential for achieving high-resolution and high-SNR imaging outcomes, particularly under sub-optimal imaging conditions. Additionally, we incorporate a 775 nm nanosecond pulsed laser for STED microscopy, which surpasses the diffraction limit and provides nanoscale spatial resolution by depleting the outer 2PE fluorescence shell through stimulated emission. By successfully integrating these advanced techniques in a single microscope platform, we present a powerful tool for investigating biological processes at the nanoscale in tissues. This combined system holds promise in unravelling complex cellular interactions and exploring subcellular structures with unprecedented detail, enabling breakthroughs in biophysics and cancer research. The ability to simultaneously acquire high-resolution images from multiple imaging modalities opens new avenues for understanding complex biological phenomena in their natural environments[4].

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ENHANCING THE RESOLUTION OF IMAGE SCANNING MICROSCOPY (ISM): SPLIT-ISM AND ITS APPLICATION TO THE IMAGING OF CELLULAR STRUCTURES

Elisabetta Di Franco[1], Paolo Bianchini[2], Giuseppe Vicidomini[3], Alberto Diaspro[2,4], Giulia Tedeschi[5], Lorenzo Scipioni[5],Enrico Gratton[5],Michelle Digman[5] & Luca Lanzanò[1,2]

[1]Department of Physics and Astronomy "Ettore Majorana", University of Catania.
[2]Nanoscopy CHT Erzelli, Istituto Italiano di Tecnologia, Genoa, Italy.
[3]Molecular Microscopy and Spectroscopy, CHT Erzelli, Istituto Italiano di Tecnologia, Genoa, Italy.
[4]DIFILAB, Department of Physics, University of Genoa, Genoa, Italy. [5]Laboratory of Fluorescence Dynamics, University of California Irvine.

Confocal microscopy is a bio-imaging technique which increases the resolution using a spatial pinhole to block out-of-focus light. In theory, the maximum resolution and optical sectioning is obtained when the detection pinhole is fully closed but this is prevented by the dramatic decrease of the signal reaching the detector. In image Scanning Microscopy (ISM) this limitation is overcome by using an array of point-detectors rather than a single detector. This, combined with pixel reassignment, increases the resolution of $\sqrt{2}$ over widefield imaging, with relatively little modification to the existing hardware of a laser-scanning microscope. Separation of photons by lifetime tuning (SPLIT)[1] is a super-resolution technique based on the phasor analysis of the fluorescent signal into an additional channel of the microscope. Here, we apply SPLIT technique to the images recorded by an ISM microscope to further improve the resolution, contrast, and SNR (SPLIT-ISM). Application of SPLIT requires an additional channel of the microscope. In SPLIT- ISM, we use the distance between the detectors and the center of the array. We apply the technique on data acquired by two different ISM setups: the SPAD array-based PRISM from Genoa Instruments[2] and the ZEISS Airyscan[3]. We evaluated the resolution improvement of both ISM systems through profile analysis and through the use of the QuICS[4] algorithm which allows us to extract three parameters related to the resolutions, contrast, and SNR of the image. Next, we applied the method to the study of chromatin organization in live cells. We studied different cell lines (HeLa, MDA-MB231 and MCF10a) that were treated to modulate the compaction/decompaction of chromatin and we evaluated the coefficient of variation of the image intensity to monitor their behaviour under the different treatments.

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MULTISCALE FLUORESCENCE IMAGING STUDY TO EXPLORE THE RELATIONSHIP BETWEEN PD-L1 AND LIPID RAFTS IN NON-SMALL CELL LUNG CANCER CELLS

Simone Civita,[a,c] Martina Ruglioni,[c] Tiziano Salvadori,[d] Sofia Cristiani,[d] Vittoria Carnicelli,[d] Serena Barachini,[e] Iacopo Petrini,[f] Matteo Mariangeli,[b,a] Irene Nepita,[a] Marco Castello,[a,g] Stefano Fogli,[c] Romano Danesi,[c] Alberto Diaspro,[a,h] Paolo Bianchini,[a,h] Barbara Storti,[i] Ranieri Bizzarri[d,i]

a Nanoscopy, Istituto Italiano di Tecnologia, Via E. Melen 83, 16152 Genova, Italy
b Department of Mathematical, Physical and Computer Sciences, University of Parma - Parma, Italy
c Unit of Clinical Pharmacology and Pharmacogenetics, Department of Clinical and Experimental Medicine, University of Pisa, Pisa, Italy
d Department of Surgical, Medical and Molecular Pathology, and Critical Care Medicine, University of Pisa, Via Roma 65, 56126 Pisa, Italy
e Department of Clinical and Experimental Medicine, University of Pisa, 56126 Pisa, Italy
f Department of Translational Research & New Technologies in Surgery and Medicine, University of Pisa and Azienda Ospedaliero Universitaria Pisana, 56100 Pisa, Italy
g Genoa Instruments s.r.l., Via E. Melen 83, 16152 Genova, Italy
h DIFILAB, Dipartimento di Fisica, Università degli Studi di Genova, Via Dodecaneso 33, 16146 Genova, Italy
i NEST, Scuola Normale Superiore and Istituto Nanoscienze-CNR, Piazza San Silvestro 12, 56127 Pisa, Italy
Simone.civita@iit.it

The membrane protein Programmed Death Ligand 1 (PD-L1) plays a pivotal role in various human tumors and has recently become a crucial target for cancer therapy, with clinical studies demonstrating the effectiveness of immunotherapeutic treatment. Besides the well-characterized immunosuppression in the PD-1/PD-L1 immune checkpoint, its subtle regulatory roles are still unclear and should be closely related to its molecular properties in different cellular contexts.

In this study, we investigated the organization of PD-L1 in the plasma membrane of a non-small cell lung cancer (NSCLC) cell line[1]. Through various fluorescence imaging techniques, including super-resolution techniques, we were able to study the spatial organization of the protein at different spatial scales, down to the nanoscale. For the first time, we revealed that the protein is predominantly associated with cholesterol-enriched raft zones of the membrane. This observation was made possible through a study of functional colocalization with key proteins present in membrane rafts. Additionally, super-resolution techniques showed that the distribution of proteins is predominantly clustered in regions with a radius of around 30-40 nm, a size consistent with the dimensions of plasma membrane raft regions. Furthermore, the application of spot variation-STED-FCS in living cells has identified a confined diffusion modality of PDL1 similar to other proteins that belong to Lipid Rafts.

Moreover, the implications of PD-L1 raft engagement could unveil unreported biological functions. Preliminary evidence[2] suggests a connection between raft engagement and the immune checkpoint associated with PD-L1/PD-1 interaction, offering insights for developing novel immuno-oncology strategies. Furthermore, the non-clustered PD-L1 pool may be linked to diverse non-immune functions. Ongoing experiments aim to elucidate these aspects further. This comprehensive exploration sheds light on the intricate spatial organization of PD-L1 and its potential implications in the development of targeted therapeutic strategies for NSCLC.

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LEVERAGING THE E222Q REPLACEMENT TO GENERATE NOVEL REVERSIBLY SWITCHABLE FLUORESCENT *AEQUOREA VICTORIA* PROTEINS FOR SUPER-RESOLUTION IMAGING

Francesco Garzella,[1] Barbara Storti,[2] Andrea Mussini,[3] Giulia Lo Gerfo,[2] Simone Civita,[3,4] Riccardo Nifosì,[2] Alberto Diaspro,[4,5] Paolo Bianchini,[4] Stefania Abbruzzetti,[3] Cristiano Viappiani,[3] Ranieri Bizzarri[1,2]

[1]Department. of Surgical, Medical and Molecular Pathology and Critical Care Medicine, University of Pisa, Pisa, Italy.
[2]NEST Scuola Normale Superiore & Istituto Nanoscienze-CNR, Pisa, Italy
[3]Department of Mathematical, Physical and Computer Sciences, University of Parma, Parma, Italy
[4]Nanoscopy and NIC@IIT, CHT Erzelli, Istituto Italiano Tecnologia, Genoa, Italy
[5]DIFILAB Department of Physics, University of Genoa, Genoa, Italy;
francesco.garzella@med.unipi.it

Reversibly photoswitchable fluorescent proteins (RSFPs) admirably combine the genetic encoding of fluorescence with the ability to repeatedly toggle between a bright and dark state, adding a new temporal dimension to the fluorescence signal. Accordingly, in the last years RSFPs have paved the way to novel applications in cell imaging that rely on their reversible photoswitching, including many super-resolution techniques such as F-PALM, RESOLFT, and SOFI that provide nanoscale pictures of the living matter. Among them, SOFI exploits the fluorescence blinking of probes under microscope illumination to generate super-resolved images from the temporal correlations of independently fluctuating fluorescent emitters (cumulants). Clearly the role of rsFPs becomes prominent in the so-called pcSOFI approach[1] that brings a $\sqrt{2}$x-2x improvement over diffraction-limited resolution by exploiting the 2nd order temporal auto- or spatial cross-cumulant of fluorescence fluctuation in each pixel. Yet many RSFPs have been engineered by a rational approach only to a limited extent, in absence of clear structure-property relationships that in most cases make anecdotic the emergence of the photoswitching. We have discovered E222Q replacement is a single photoswitching mutation since it restores the intrinsic cis-trans photoisomerization properties of the chromophore in otherwise non-switchable Aequorea proteins of different color and mutation pattern (Q-RSFPs)[2,3]. Our findings link indissolubly photoswitching and Q222 presence, by a simple yet elegant scenario: largely twisted chromophore structures around the double bond (including hula-twist configurations) are uniquely stabilized by Q222 via H-bonds. Likely, these Hbonds subtly modulate the electronic properties of the chromophore, enabling the conical intersection that connects the excited cis to ground trans chromophore[2]. Remarkably, analysis of photoswitching by fast spectroscopy revealed multiphase kinetics related to the peculiar protonation pattern of the protein. By applying E222Q to simple derivatives of the Enhanced Green Fluorescent Protein we generated a palette of green and yellow emitting Q-RSFPs that are tailored to several techniques addressing intracellular settings at nanoscale, such as photochromic FRET/anisotropy, SOFI, and F-PALM. This research has been partially funded by the project PRIN 2022RRFJC4 "Novel protein-based Genetically-Encoded Fluorescent Indicators (GEFI) for Functional Super-Resolution Imaging of Biomolecular Activities in Living Cells [GEFInder]"

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SINGLE-PHOTON MICROSCOPY TO STUDY STRESS GRANULES

Eleonora Perego,[1] Sabrina Zappone,[1,2] Francesco Castagnetti,[1] Davide Mariani, [1] Erika Vitiello, [1] Jakob Rupert, [1,3] Elsa Zacco, [1] Gian Gaetano Tartaglia, [1,3] Irene Bozzoni, [1,3] Eli Slenders[1] and Giuseppe Vicidomini[1]

1Molecular Microscopy and Spectroscopy, Istituto Italiano di Tecnologia, Genova, Italy
2DIBRIS, University of Genova, Genova, Italy
3Department of Biology and Biotechnologies, Sapienza University of Rome, Rome, Italy
sabrina.zappone@iit.it

Among all the strategies cells employ to adapt to stress, the formation of micron-scale liquid-like compartments known as stress granules (SGs) stands out as one of the most studied. Acting as reservoirs, SGs concentrate proteins and nucleic acids when external stressors disrupt their structure and function. The aberrant and persistent formation of SGs induced by mutations, such as mutations affecting FUS protein, has been related to neurodegenerative diseases[1]. While conventional imaging techniques have provided insights into SG formation at the cellular scale, only spectroscopy methods, such as fluorescence lifetime correlation spectroscopy (FLFS), can reveal dynamics within these compartments. Equipping a confocal microscope with a single-photon array detector, we developed a framework to simultaneously monitor diffusion, mobility modes (free, domain-confined or meshwork-constrained diffusion), interactions, and micro-environment organization of molecules involved in the SG turnover[2,3]. We applied our method to investigate the dynamics of two players in SGs, G3BP1 and (wild-type and mutated) FUS proteins, across various spatial and temporal scales. Our multi-parameter analysis revealed distinct diffusion and mobility modes of the two proteins, despite both being recruited within SGs[3]. This sheds light on the ability of the FLFS-based framework to capture the heterogeneity of molecules orchestrating the same biomolecular process and provide insights into the molecular mechanisms underlying the cellular stress response.

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AFM-BASED INVESTIGATION OF CHOLESTEROL IMPACT ON NANOMECHANICAL PROPERTIES OF BIOMIMETIC PLASMA MEMBRANES

Luca Puricelli[1], Elena Ferraguzzi[2,3], Pietro Parisse[4], and Loredana Casalis[2]

[1] Area Science Park, 34149 Padriciano (TS), Italy
[2] Elettra Sincrotrone Trieste, 34149 Basovizza (TS), Italy
[3] Department of Physics, University of Trieste, 34127 Trieste, Italy
[4] IOM-CNR, 34149 Basovizza (TS), Italy
luca.puricelli@areasciencepark.it

In the last decades, the research interest on biophysical topics for theranostic purposes has been growing. In particular, keen attention has been recently dedicated to the study of cell plasma membranes (PM), which represent the ultimate cellular barrier and pivotal step of interaction with extracellular systems, such as vesicles, pathogens and drugs. Among the great biochemical heterogeneity of PM, cholesterol is currently acknowledged as one of the fundamental players regulating membrane stability, dynamics and mechanical properties[1,2]. These, in turn, are widely recognized as an important biomarker of the patho-physiological state of biological specimens, thus being worth a quantitative characterization, in correlation with other relevant biophysical properties of the system.

In order to investigate these topics, here we will present our preliminary AFM-based nanomechanical measurements on in-vitro biomimetic models of PM, namely supported lipid bilayers (SLB), including some of the most representative lipids (DOPC, DSPC, SM) together with different cholesterol concentrations. In particular, we will highlight the mechanical contrasts between the diverse coexistent phases typically observed in PM, correlating them with the variations induced by different cholesterol concentrations. Eventually, we will discuss the main theoretical and experimental issues to be faced in order to get a robust and reproducible nanomechanical characterization of these complex biological systems.

Altogether, our results aim to get a more detailed insight about the key biophysical properties ruling cellular membrane functioning, as a first step for potential applications in the theranostic field, such as novel drug-delivery strategies or cancer therapies targeting the membrane lipidic composition[3].

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BIOFISICA MOLECOLARE

THERMODYNAMIC AND KINETIC EFFECTS ON THE ACTIVITY AND SELECTIVITY OF ANTIMICROBIAL PEPTIDES

*F. Carneri[1], E. Franci[1], C. Troiano[1], G. Giaquinto[1], A. Leoci[1], D. Roversi[1], M. R. Loffredo[2], Y. Park[3], A. Toto[2], S. Gianni[2], M. L. Mangoni[2], H. Franzyk[4], L. Stella[1, *]*

[1] Deparment of Chemical Science and Technologies, University of Rome Tor Vergata,00133 Rome, Italy;
[2]Deparment of Biochemical Sciences, Sapienza University of Rome, Istituto Pasteur Italia-Fondazione Cenci Bolognetti, 00185 Rome, Italy.
[3]Department of Biotechnology, Chosun University, 501-759 Gwangju, Korea.
[4]Department of Drug Design and Pharmacology, University of Copenhagen, DK-2100 Copenhagen, Denmark
*stella@stc.uniroma2.it

Biophysical studies of the interaction of antimicrobial peptides (AMPs) with artificial membranes are pivotal in developing models of the mechanisms of pore formation. However, quantitative data on the behaviour of AMPs in real cells are scant. We developed assays allowing the thermodynamic and kinetic characterization of peptide interaction with live bacterial cells [1, 2, 3, 4, 5]. Our results clarified some key aspects of AMP function but also led to several new questions. For instance, a concentration range wherein some bacteria are killed, while others survive, is usually present. Within this interval (often called “mutant selection window”, or MSW) resistance can develop. Phenotypic or genetic differences between individual cells could be the origin of the heterogeneous bacterial response to AMPs. However, this explanation cannot apply to the similar behaviour observed on liposomes, which are extremely homogeneous in terms of size and composition [6]. Our result indicate that the thermodynamic equilibrium of water/membrane partition equilibrium plays a key role in the heterogeneous response observed in liposomes. When the membrane-bound peptide concentrations are considered, rather than total concentrations, the MSW is essentially abolished. However, in situations where two cell populations (bacteria and host cells) or two different liposome types are present, thermodynamics alone does not explain the observed selectivity, suggesting that kinetic effects could be at play. For this reason, we are currently studying the kinetics of peptide/cell interactions. Our findings indicate that peptides bind to bacterial membranes in less than a second, perturb them within a few minutes, causing bacterial death, and ultimately, localize inside the cells. Overall, a combined thermodynamic and kinetic characterization provides a unique perspective on the activity and selectivity of AMPs.

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MONITORING MOLECULAR ORDERING IN LIQUID-LIQUID PHASE SEPARATION PROCESSES

Giuseppe De Luca[1] *Valeria Vetri*[2] *Giuseppe Sancataldo*[2]

1. Department Biological, Chemical and Pharmaceutical Sciences and Technologies (STEBICEF), University of Palermo, Viale delle Scienze, 16, 90128, Palermo, Italy
2. Department of Physics and Chemistry – Emilio Segrè, University of Palermo, Viale delle Scienze, 18, 90128, Palermo, Italy

giuseppe.deluca04@unipa.it

The analysis of Liquid-Liquid Phase Separation (LLPS) in model protein solutions may provide insight into spontaneous phase separation mechanisms in biological systems. Within the cell, LLPS underlies the formation of membrane-less organelles, liquid biomolecular coacervates, in which specific biochemical processes occur[1]. LLPS is a complex phenomenon which implies a delicate balance between enthalpic and entropic contributions. The role of solvent, and particularly water molecules ordering, is recognized as crucial in orchestrating phase separation, even if it challenging to elucidate[2]. In this context, the use of 6-acetyl-2-(dimethylamino) naphthalene (ACDAN), a hydrophilic fluorescent dye that monitors the dipolar relaxation of the solvent, constitutes a promising tool to dynamically probe the ordering of the aqueous phase[3].

Here, we present experimental study on the LLPS process in a model system composed of an aqueous solution containing Bovine Serum Albumin (BSA) and polyethylene glycol (PEG) as a molecular crowder. The system was characterized by means of turbidity measurements, steady-state fluorescence, and time-resolved fluorescence techniques. Results show that the sample is homogeneous at high temperature and undergoes LLPS by lowering the temperature. In the analysed conditions, the critical temperature for phase transition is found to be correlated to the crowder concentration. To explore the molecular order of the aqueous phase and thus the role of water structural organization, a chaotropic agent and a kosmotropic agent were added to the sample, and the transition temperature analysed as functional of their concentration. The disordering/ordering effects of these molecules results in an increase/decrease of the transition temperature, indicating the crucial role of water H-bonding network in the process. To deeply explore how the transition affects the ordering of the system, ACDAN was used. ACDAN fluorescence quantified by GP (Generalised Polarisation) was used to explore the dipolar relaxation in the sample, revealing an overall ordering of the system as result of the phase transition. Furthermore, by means of FLIM (Fluorescence Lifetime Imaging Microscopy) measurements we were able to discriminate differences in the ordering level of diluted and concentrated phase in the LLPS sample revealing an increased order in the BSA concentrated phase.

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INVESTIGATION ON MMACHC-R161Q PATHOLOGICAL MUTANT FROM CBLC DISEASE, A RARE METABOLIC DISORDER OF VITAMIN B12 METABOLISM

Lisa Longo[1,2], Rita Carrotta[1], Maria Assunta Costa[1], Rosa Passantino[1], Maria Rosalia Mangione[1], Loredana Randazzo[1], Maria Grazia Ortore[3], Michela Bollati[1], Matteo De Rosa[1], Mario Milani[1], Adrian Velazquez Campoy[4], Marco Tutone[2], Vincenzo Martorana[1], Silvia Vilasi[1]

1. Istituto di Biofisica, Consiglio Nazionale delle Ricerche, Italy
2. Dipartimento Scienze e Tecnologie Biologiche Chimiche e Farmaceutiche, Università degli Studi di Palermo, Italy
3. Dipartimento di Scienze della Vita e dell'Ambiente, Università Politecnica delle Marche, Ancona, Italy
4. Institute for Biocomputation and Physics of Complex Systems (BIFI), Zaragoza, Spain

The cblC disease is a rare inborn disorder of the vitamin B12 (cobalamin, Cbl) metabolism characterized by combined methylmalonic aciduria and homocystinuria. The clinical consequences are devastating and, even when early treated with current therapies, the affected children manifest symptoms involving vision, growth, and learning[1]. The molecular genetic cause of the disease was found in the mutations of the gene coding for MMACHC, a 282 amino acid protein that transports and processes the various forms of Cbl[2]. Although the crystal structure of the wild-type protein is available[3,4], many molecular features of MMACHC physiopathology remain to be understood and a systematic study on the effect of each specific mutation on the resulting protein is still lacking. Here we present the biophysical characterization of wild type MMACHC and a variant, p.R161Q, resulting from the most common missense pathological mutation found in CblC patients. By using a biophysical approach, we investigated the stability of the two proteins and their ability to bind and transform the vitamin B12[5], and to assemble in a dimeric structure. Moreover, we evaluated whether drug-like molecules identified by computational methods, or non-specific stabilizers (osmolytes) could restore the functionality in MMACHC mutant. Overall, our results reveal how a biophysical approach based on the complementarity of computational and experimental methods can offer new insights in the study of the specific effects of the pathological cblC mutation and help prospecting new routes for the cblC treatment.

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COMBINING ADVANCED AFM AND FLUORESCENCE MICROSCOPY TO STUDY THE ANTIVIRAL EFFECTS OF HYPERICIN.

Matteo Mariangeli[1,2], Silvia Dante[3], Patricia Pedraz[4], Pietro Delcanale2, Stefania Abbruzzetti[2], Alberto Diaspro[1,5], Cristina Flors[4], Cristiano Viappiani[2], Paolo Bianchini[1,2,5]

[1]Nanoscopy and NIC@IIT, Istituto Italiano di Tecnologia, Genoa, Italy
[2]Department of Mathematical, Physical and Computer Sciences, University of Parma, Parma, Italy
[3]Materials Characterization Facility, Istituto Italiano di Tecnologia, Genoa, Italy
[4]IMDEA Nanociencia, Madrid, Spain
[5]DIFILAB, Department of Physics, University of Genoa, Genoa, Italy
matteo.mariangeli@iit.it

Hypericin (Hyp) is a small, lipophilic compound well-known for its fluorescent and photosensitizing capabilities, recognized as an effective antiviral agent, with or without light exposure, as concerns enveloped viruses (*1*). Our study aims to uncover the mechanisms behind Hyp's virucidal ability by using a combination of fluorescence microscopy and atomic force microscopy (AFM) for a comprehensive understanding of the nanoscale structure, mechanics, and chemical properties(*2*),(*3*). For this purpose, phase-separated supported lipid bilayers (SLBs) (*4*) that simulate the lipid composition of enveloped viruses (*5*) were used as a model.

AFM topography uncovered significant morphological changes in SLBs incubated with Hyp under light exposure. These changes are thought to be a result of Hyp's photodynamic activity, which triggers lipid rearrangements and subsequent structural alterations in the SLB. Given the rapid occurrence of these changes, we employed FastScan AFM to gain a more detailed understanding of the dynamic processes involved. Furthermore, by correlative confocal fluorescence imaging and AFM, we successfully determined that Hyp exhibits a distinct binding affinity to different lipid phases: it is believed that the presence of cholesterol in the SLB plays a role in this process. Beyond the effects induced by light, Atomic Force spectroscopy has revealed that Hyp alters the nanomechanical properties of SLBs, leading to increased stiffness. As a result, Hyp could hinder viral entry (*6*),(*7*) providing a reasonable explanation for its antiviral action in the dark.

Ultimately, combining fluorescence microscopy with AFM allowed an extensive picture of Hyp's behavior in viral-mimetic membranes, providing insights on its antiviral activity.

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ROLE OF CHOLESTEROL AS LIPID ORDER/FLUIDITY MODULATOR IN BIOMIMETIC PLASMA MEMBRANES

Elena Ferraguzzi[1,5] *Nishu Kanwa*[2] *Luca Puricelli*[3] *Yusuf Qutbuddin*[2] *Petra Schwille*[2] *Pietro Parisse*[4], *and Loredana Casalis*[5]

[1]Department of Physics, University of Trieste, 34127, Trieste, Italy
[2]Max Planck Institute of Biochemistry. Am Klopferspitz 18 82152 Martinsried.
[3]AREA Science Park - Padriciano, 99 34149 Trieste – Italy
[4]Istituto Officina dei Materiali – CNR s.s 14 km 163.5 in Area Science Park 34149 Basovizza- Trieste-Italy
[5]Elettra-Sincrotrone Trieste S.C.p.A SS 14 Km 163.5 in Area Science Park 34149 Basovizza, Trieste, Italy

Lipid rafts (LR) are nano-sized plasma membrane domains (10-200 nm) involved in controlling numerous vital cellular processes[1]. They are rich in cholesterol, which plays a fundamental role in modulating membrane properties as order and fluidity, regulating transport and concentration of proteins within the membrane lipid bilayer, concurring to promote their specific functions. Due to their small size and fast dynamics, LR are difficult to be studied in physiological systems. The present study investigates the influence of cholesterol on LR formation and its impact on membrane fluidity in biomimetic membrane systems. As a model, we chose a 4-component lipid composition (DOPC: DSPC:SM and different cholesterol concentration (5mol% and 33mol%) mimicking cell membrane physiological conditions. Atomic force microscopy (AFM) measurements, conducted under controlled temperature, unveiled a correlation between cholesterol levels and lipid raft height/dimensions, while fluorescence recovery after photobleaching (FRAP) measurements provided quantitative insights into membrane diffusion dynamics. This study underscores the utility of model membrane systems in elucidating essential physicochemical parameters, thereby advancing our understanding of complex biological processes occurring at the membrane.

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IRON DEPOSITION IN FERRITIN CORES

Caterina Ricci,[1] Flavio Di Pisa,[2] Anna Moroni,[3] Elena Del Favero[1]

[1]BIOMETRA, Università Statale di Milano -via Fratelli Cervi 93, Milano, Italy
[2]IBF-CNR, via Celoria 26, Milano, Italy
[3]Dipartimento di Bioscienze, Uiversità Statale di Milano, via Celoria 26, Milano, Italy
caterina.ricci@unimi.it

Iron is quantitatively the most present metal in the body and assists many biological reactions, such as mitochondrial respiration, cell proliferation and protein synthesis. As well, it could be highly toxic by facilitating the formation of free radicals. In cases of iron deficiency and overload, serum ferritin serves a critical role in both diagnosis and management in cellular environments (1,2). Anomalous accumulation of iron in the brain is the hallmark of different pathologies, such as the late-onset, autosomal dominantly transmitted condition called Neuroferritinopathy (3).
The hollow sphere structure of the protein allows the iron to be stored, and the empty protein can collect iron atoms until the formation of a mineral nucleus of hydrated ferric oxide. Thanks to its trapping ability, this protein may have also promising biotechnological applications in the field of nanomedicine, from nanovectors for the transport of drugs, to magnetosensitive probes (4). Several efforts were made in the recent years to engineer genetically encoded proteins able to respond to magnetic stimulations (5).
In our study, the structural properties of the protein in aqueous solution were characterized by means of different techniques (Small Angle X-ray Scattering, Dynamic Light Scattering and Small Angle Neutrons Scattering). Human wild type and mutated ferritin and ferritin from Pyrococcus Furiosus were expressed, suitably purified and studied at different concentrations of iron to apperceive the storage mechanisms of proteins in various conditions, from physiological ones to those related to biotechnological applications.

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FROM WASTE TO RESOURCE: HOP EXTRACT AND ITS BIOACTIVE COMPOUNDS CAN INHIBIT AMYLOID AGGREGATION

Eleonora Mari[1], Maria Rosalia Mangione[1], Silvia Vilasi[2] and Maria Grazia Ortore[1]

1. Dipartimento di Scienze della Vita e dell'Ambiente, Università Politecnica delle Marche, Via Brecce Bianche, 60100 Ancona, Italy
2. Istituto di Biofisica, CNR, Via Ugo La Malfa, Palermo

e.mari@pm.univpm.it

Misfolding and amyloid aggregation of proteins have attracted scientific interest from several decades, due to their link with several neurodegenerative diseases. It is well known that some natural compounds can interfere with amyloid aggregation by preventing the formation of mature fibrils1,2. Hence, selected natural compounds may be introduced into the diet, to prevent the main biochemical mechanisms responsible for the amyloid aggregation3. Also, since agricultural waste is often rich in precious natural molecules such us polyphenols, flavonoids, and procyadines, we produced and optimized extracts from hop leaves.

We investigated both the hop extracts and some of their bioactive compounds (saponins, xanthohumol and α-humulene) influence on fibrillation of human insulin and of amyloid beta peptide.

UV-Visible absorption and fluorescence spectroscopy, using Congo Red and Thioflavin T as amyloid specific probes4, provided the β-structure during fibrillation. To study the overall structural features and size of aggregates we performed synchrotron Small Angle X-ray Scattering and Dynamic Light Scattering experiments, while to monitor secondary structure changes (from α-helix to β-sheets) we performed Circular Dichroism experiments.

Our experimental results prove that the hop extract interferes with the amyloid aggregation by preventing or reducing the formation of fibrils. Cytotoxicity has been tested on SH-SY5Y neuroblastoma cells, too3.

The evaluation of single bioactive compounds effects compared with the whole hop extract ones, suggests possible application of extracts for biomedical purposes.

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CHOLESTEROL-CONTAINING LIPOSOMES DECORATED WITH AU NANOPARTICLES AS MINIMAL TUNABLE FUSION MACHINERY

Ester Canepa[1,2] *Davide Bochicchio*[1], *Giorgia Brosio*,[1] *Paulo Henrique Jacob Silva*,[3] *Francesco Stellacci*[1], *Silvia Dante*[2], *Giulia Rossi*[1], *Annalisa Relini*[1]

[1]Department of Physics, University of Genoa, Via Dodecaneso 33, Genoa 16146, Italy
[2]Materials Characterization Facility, Istituto Italiano di Tecnologia, Via Morego 30, Genova 16163, Italy
[3]Institute of Materials Science & Engineering, École Polytechnique Fédérale de Lausanne, Lausanne 1015, Switzerland
ester.canepa@edu.unige.it – ester.canepa@cbni.ucd.ie

Membrane fusion is essential for the basal functionality of eukaryotic cells. Synaptic transmission, membrane trafficking, and subcellular cargo transport are some physiological events involving rapid and selective membrane fusion. Defects in membrane fusion machinery may result in severe biological damage, including disorders in neuronal synapses. In physiological conditions, fusion events are regulated by a wide range of highly specialised proteins, operating with finely tuned local lipid composition and ionic environment. Fusogenic proteins, assisted by membrane cholesterol and calcium ions, provide the mechanical energy necessary to achieve vesicle fusion in neurotransmission. Similar cooperative effects must be explored when developing novel supramolecular systems mimicking controlled membrane fusion. Here we show that synthetic liposomes composed of cholesterol and 1,2-dioleoyl-sn-glycero-3-phosphocholine, and decorated with membrane-embedded amphiphilic Au nanoparticles, can act as a minimal tunable fusion machinery.1 Our Au nanoparticles have a monodisperse core diameter of 2.4 nm and are known to spontaneously and non-disruptively interact with the liposome lipid bilayer.2-4 Liposome fusion is triggered by divalent ions (Ca2+ or Mg2+), while the number of fusion events dramatically changes with, and can be finely tuned by, the liposome cholesterol content (0÷40 mol%). We combine dissipative quartz-crystal-microbalance (QCM-D), real-time fluorescence assays (vesicle content and FRET-based lipid mixing), and small-angle X-ray scattering (SAXS) with molecular dynamics (MD) at coarse-grained (CG) resolution, revealing new mechanistic details on the fusogenic activity of amphiphilic Au nanoparticles and demonstrating the ability of these synthetic nanomaterials to induce controlled vesicle fusion regardless of the divalent ion used. The results provide a novel contribution to developing new artificial fusogenic agents for next-generation biomedical applications that require tight control of the rate of fusion events (e.g., artificial synapses and targeted drug delivery).

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BLACK PHOSPHORUS NANOMATERIALS FOR NEW THERAPEUTIC STRATEGIES

Carla Caponio[1], Agata Costanzo[1], Serena Coiai[1], Francesca Cicogna[1], Emanuela Pitzalis[1], Silvia Borsacchi[1], Giulia Lorenzetti[1], Emilia Bramanti[1], Alessia Papalini[2], Antonella Battisti[2], Antonella Sgarbossa[2], Elisa Passaglia[1]**

1Istituto di Chimica dei Composti Organometallici - CNR, Via Moruzzi 1, 56124 Pisa, Italy.
2 NEST, Scuola Normale Superiore and Istituto Nanoscienze - CNR, Piazza S. Silvestro 12, 56127 Pisa, Italy
antonella.sgarbossa@nano.cnr.it

Among 2D materials, black Phosphorus (bP) monolayers, known as Phosphorene, have attracted recent scientific attention for biomedical applications due to its unique characteristics.
Few-layered bP nanosheets have been shown to be effective photosensitizers exhibiting a broad-spectrum light absorption ranging from the UV light to the near-infrared region, producing singlet oxygen ($^1O_2$) with a high quantum yield. In addition to these favorable properties, bP-nanosheets are biocompatible and nontoxic materials, which is the essential criteria for i*n vivo* applications.
Phosphorene has been studied and proposed for many biomedical applications, such as bioimaging, biosensing, and theranostics. Recently, studies on its potential antimicrobial applications, especially against antibiotic-resistant pathogens, have been gaining growing attention. In addition, direct effects of inhibition and disintegration of amyloid fibrils associated with the development of various neurodegenerative diseases (e.g. Alzheimer's disease) and metabolic disorders (e.g. diabetes) have been reported. The current study is focused on the preparation of phosphorene suspensions for biomedical applications using black phosphorus liquid phase exfoliation. To achieve this goal, two liquid exfoliating media were employed: deionized water (biocompatible and safe) and Cyrene, a new generation bio-based and potentially biocompatible solvent that has not been used previously for liquid phase exfoliation of bP. The obtained bP nanoflakes suspensions have been characterized and their stability over time as well as their photoactivity have been investigated. Preliminary data on the potential biomedical application of bP-nanosheets to inhibit the insulin self-assembly into amyloid aggregates as well as to cause fibrils disassembling through simple incubation or photoactivity, are presented.

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MICROSCOPIC INSIGHTS INTO REARRANGEMENT OF CHROMATIN DOMAINS AND EPIGENETIC DYNAMICS DURING ADIPOCYTE DIFFERENTIATION

Lama Zeaiter[1,2], Francesca Baldini[2], Ali Dabbous[3], Lisa Cuneo[2,4], Paolo Bianchini[2], Laura Vergani[1], and Alberto Diaspro2,4

1 DISTAV, Department for the Earth, Environment and Life Sciences, University of Genoa, Genoa, Italy
2 Nanoscopy, Istituto Italiano Tecnologia, Genoa, Italy
3 DITEN Department of Electrical, Electronic and Telecommunication Engineering, University of Genoa, Genoa, Italy
4 DIFILAB Department of Physics, University of Genoa, Genoa, Italy

Lama.zeaiter@iit.it

Inside the nucleus, chromatin domains are arranged non-randomly within the volume, and their ordered spatial organization participates in the regulation of genome functions also through epigenetic modifications. White adipose tissue (WAT) is the pivotal regulator of whole body energy homeostasis, acting both as physiological fat storage and endocrine organ. Our focus was on understanding the structural and functional modifications occurring in the chromatin organization as pre-adipocytes progress through adipogenesis into mature adipocytes. Utilizing a cellular model of adipogenesis consisting of the murine 3T3-L1 cell line, the rearrangement of the *in situ* chromatin was investigated by employing super resolution imaging techniques such as confocal and stimulated emission depletion (STED) microscopy, complemented by immunoassays and molecular biology methods. This multifaceted approach enabled us to map out the changes in the spatial organization of chromatin domains within the nucleus being associated to adipocyte maturation, In particular, we highlighted the presence of concentric patterns of chromatin distribution from the nuclear center to the periphery, which are often associated with the nuclear lamina. Our results unveil a significant remodeling of nuclear architecture during the transition from pre-adipocytes to mature adipocytes. Specifically, as pre-adipocytes cells assume a mature phenotype, we observed rearrangements in both DNA methylation and histone acetylation patterns, as well as in chromatin compaction, indicating that a profound reorganization of chromatin domain distribution sustain the adipogenesis process.

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HYDROPHOBIC GATING IN NANOPORES AND ION CHANNELS. FROM LIPID-MEDIATED MODULATION IN BK CHANNEL TO MEMRISTORS DESIGN FOR NEUROMORPHIC APPLICATIONS

G. Di Muccio, G. Paulo, A. Giacomello*

Sapienza University of Rome, Rome, Italy
giovanni.dimuccio@uniroma.it

Under specific conditions of surface chemistry and geometry, a vapor bubble can spontaneously form within hydrophobic nanopores at temperatures and pressures where this process wouldn't be possible in bulk water[1]. Hydrophobic gating controls the flow of ionic current through a nanopore by hindering or allowing it based on the pore's wetness or dryness, and it's relevant in both biological and synthetic pores[2]. The change in ionic conductance brought on by drying and wetting is reversible and occurs randomly in small pores, and its dynamics can range from short to long timescales depending on the pore's properties and external voltage. The large separation of spatial and temporal scales calls for the development and implementation of atomistically informed analytical models, based on enhanced sampling techniques[3]. In our group, we developed and implemented a computationally effective method to investigate the wetting and drying properties of different biological transmembrane proteins, ranging from the BK ion channel to engineered FraC nanopore.

On the biological side, our methodology allowed us to unveil an unexpected – but apparently fundamental -- role of annular lipids in BK gating machinery[4], paving the way for future studies aimed at exploring therapeutic interventions targeting these mechanisms.

On the technological side, our analysis shows that biological nanopores have the perfect geometrical properties to exploit hydrophobic gating in nanofluidic achievable devices. As proof of principle, we demonstrate that an engineered FraC nanopore with a hydrophobic mutation in its constriction acts as a "memristor", *i.e*. whose conductance displays memory behaviours, as predicted by our theoretical model. These findings open the door for a new generation of nanofluidic devices based on hydrophobic nanopores, which can have applications in neuromorphic computing[5].

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DIRECT RECORDINGS OF ELECTRON CURRENTS IN PLANT VACUOLES MEDIATED BY CYTOCHROME B561A ACTIVITY

Antonella Gradogna[1], Laura Lagostena[1], Sara Beltrami[2], Edoardo Tosato[2], Cristiana Picco[1], Joachim Scholz-Starke[1], Francesca Sparla2, Paolo Trost[2] and Armando Carpaneto[1,3]

1 Institute of Biophysics - CNR, Via De Marini 6, 16149 Genova, Italy
2 Department of Pharmacy and Biotechnology (FaBiT) - University of Bologna, Via Irnerio 42, 40126 Bologna, Italy
3 Department of Earth, Environment and Life Sciences (DISTAV) - University of Genoa, Viale Benedetto XV 5, 16132 Genova, Italy
armando.carpaneto@unige.it

Ascorbate (Asc) is a versatile antioxidant of plant cells, whose redox activity depends on the ratio with its one-electron oxidation product monodehydroascorbate (MDHA). The cytoplasm contains high concentrations of Asc and soluble enzymes that can regenerate Asc from its oxidized forms. The vacuole, the largest compartment in mature cells, also contains Asc, but no soluble Asc-regenerating enzymes. In this work we show that an electron transporter, which works as a reversible, Asc-dependent transmembrane MDHA oxidoreductase, is localised on the membrane of vacuoles from Arabidopsis mesophyll cells.

Electron currents[1], detected 39 years after the first patch-clamp potassium current recordings in plant cells, were measured on isolated vacuoles and found to depend on the availability of Asc (electron donor) and ferricyanide or MDHA (electron acceptors) on opposite sides of the membrane. Electron currents were mediated by the tonoplast redox protein cytochrome b561 isoform A (CYB561A). The apparent affinities for Asc of the luminal (4.5 mM) and cytoplasmic sites (51 mM) reflected the physiological Asc concentrations in these compartments. In mutant plants with impaired CYB561A expression there were no detectable trans-tonoplast electron currents; under high-light stress, mutants showed strong accumulation of leaf anthocyanins, suggesting a CYB561A redox-modulation on the typical anthocyanin response to excessive illumination.

Experimental data could be described by an extension of the Michaelis–Menten equation, derived from a simplified kinetic model, shedding light on the complex relationships among the electron acceptor and electron donor in controlling CYB561A activity.

Furthermore, we recently conducted measurements of electron currents[2] for another protein belonging to the cytochrome b561 family. This protein is located on the plasma membrane of Arabidopsis thaliana cells and is implicated in the reduction of extracellular iron.

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COMPLEMENTARY AND ORTHOGONAL BIOPHYSICAL APPROACHES DISCLOSE STRUCTURAL AND FUNCTIONAL DIFFERENCES IN HUMAN OSTEOCALCIN AS A FUNCTION OF ITS CARBOXYLATION DEGREE

Antonino Natalello,[1] Diletta Ami,[1] Carlo Santambrogio,[1] Jacopo Vertemara,[1] Federica Bovio,[1] Andrea Santisteban-Veiga,[2,3] Juan Sabín,[2,3] Giuseppe Zampella,[1] Rita Grandori,[1,4] Laura Cipolla[1]

[1]Department of Biotechnology and Biosciences, University of Milano-Bicocca, Piazza della Scienza 2, 20126 Milan, Italy.
[2]*AFFINImeter Scientific & Development team, Software 4 Science Developments, Santiago de Compostela, 15782, Spain.*
[3]*Applied Physics Department, University of Santiago de Compostela, Santiago de Compostela 15782, Spain.*
[4]*Institute for Advanced Simulations, Forschungszentrum Juelich, 52428 Juelich, Germany*
antonino.natalello@unimib.it

Osteocalcin (OC) is the main non-collagenous protein found in the bone extracellular matrix, which also acts as a bone-derived factor that regulates glucose metabolism, male fertility, acute stress response, endurance exercise, adrenal steroidogenesis, and blood pressure. In human OC, three conserved glutamic acid residues are partially or fully converted to γ-carboxyglutamic acid, at position 17, 21 and 24. This reversible, vitamin K-dependent, γ-carboxylation modulates OC release from bones and its hormonal roles. A complete understanding of OC roles and structure-activity relationships is still lacking, as only uncarboxylated and a few differently carboxylated variants have been considered so far. To fill this lack of knowledge, a comprehensive experimental and computational investigation of the structural properties and calcium-binding activity of all the eight OC variants is reported here. In particular, we employed circular dichroism (CD) spectroscopy for secondary structure, native mass spectrometry (nMS) for stoichiometry and species distributions, isothermal titration calorimetry (ITC) for thermodynamic signatures and computational approaches for structural interpretation.
Such a comparative study indicates that the carboxylation sites are not equivalent and differently affect OC structure and the interaction with calcium, properties that are relevant for the modulation of OC functions. This study also discloses cooperative effects and provides a structural and mechanistic interpretation. In particular, carboxylation at position 24 resulted to be that with the highest affinity for calcium and was crucial for stabilizing the protein α-helical structures. Carboxylation at position 21 showed a cooperative effect, as its interaction with calcium favors binding to residue 17. The disclosed peculiar features of each carboxylated proteoform strongly suggest that considering all the eight possible OC variants in future studies may help rationalise some of the conflicting hypotheses observed in the literature. This research was funded by Fondazione Cariplo (grant number: 2018-0458).

SEGMENTED FLUORESCENCE CORRELATION SPECTROSCOPY (FCS) ON A COMMERCIAL MICROSCOPE TO STUDY DYNAMICS IN LIVE CELLS

Elisa Longo,[1] Anna P. Privitera,[1] Silvia Scalisi, [1] and Luca Lanzanò[1,2]

[1]Department of Physics and Astronomy "Ettore Majorana", University of Catania, Catania, Italy
[2]Nanoscopy, CHT Erzelli, Istituto Italiano di Tecnologia, Genoa, Italy
elisa.longo@dfa.unict.it

Fluorescence Correlation Spectroscopy (FCS) is a powerful method for investigating molecular dynamics and interactions in biological systems. Typically, FCS is performed on specialized confocal set-ups where the excitation laser is focused on a point of the specimen. Subsequently, the fluorescence intensity fluctuations are acquired, and an autocorrelation function (ACF) is generated by the dedicated software. The application of FCS in cells is more challenging due to the complexity of the cellular environment. In this respect, it has been shown that analysis of the data in short temporal segments can be very useful to increase the accuracy of FCS measurements inside cells. This study demonstrates the extraction of segmented FCS data using a commercial laser scanning microscope, even without a dedicated FCS module. Our approach involves acquiring data in line or raster scan mode on a Leica SP8 confocal microscope followed by export and processing using a custom script in Matlab. Through segmentation of the data, we extract the ACF and derive important biophysical parameters, such as the diffusion coefficient, within different zones of the specimen. Two acquisition methods are presented: X segmentation, with the highest temporal resolution for fast fluorophore diffusion measurement, and Y segmentation, more suitable for measuring the slower dynamics within sub-cellular structures. First, we validate the method using solutions of fluorophores of different size. Next, we measure the diffusion coefficients of untagged GFP (X segmentation) and a PARP-1 chromobody tagged with RFP (Y segmentation) in the nucleus of Hela cells. Different nuclear regions are analyzed based on intensity variations, revealing slower diffusion in the nucleolus compared to the nucleoplasm, because of the higher crowding of nucleolar compartments and/or binding of the probe. Finally, the method is applied to investigate PARP1 dynamics at sites of laser-induced DNA damage. Following irradiation of a small region (2.5 μm x 0.2 μm) we observe a rapid (in the order of seconds) accumulation of the PARP1 protein on the lesion. Performing segmented FCS, we measure the diffusion coefficient inside and outside the damaged chromatin region, characterizing the mobility of PARP1 during the DNA damage repair process.
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NANOALGOSOMES AS DRUG DELIVERY SYSTEM FOR NEUROPROTECTIVE DRUGS IN C. ELEGANS MODEL OF SPINAL MUSCULAR ATROPHY (SMA)

Angela Paterna,[1] Pamela Santonicola,[2] Estella Rao,[1] Samuele Raccosta,[1] Sabrina Picciotto,[3] Giorgia Adamo,[3] Antonella Bongiovanni,[3] Elia Di Schiavi,[2] and Mauro Manno[1]

[1] National Research Council of Italy (CNR), Cell-Tech Hub and Institute of Biophysics, Via Ugo La Malfa 153, 90146 Palermo, Italy
[2] National Research Council of Italy (CNR), Institute of Biosciences and Bio-Resources, Via Pietro Castellino 111, 80131 Napoli, Italy
[3] National Research Council of Italy (CNR), Cell-Tech Hub and Institute for Research and Biomedical Innovation, Via Ugo La Malfa 153, 90146 Palermo, Italy
angela.paterna@ibf.cnr.it

A challenging issue in the field of nanotechnology is the development of smart nanocarriers aimed to improve the effectiveness of drugs by addressing issues like limited targeting, excessive side effects, degradation, and the need for high doses.
Extracellular vesicles (EVs) are a natural heterogeneous group of lipid nanoparticles secreted by cells and involved in multiple physiological and pathological processes. Previous research has highlighted nanoalgosomes, EVs obtained from the microalgae *Tetraselmis chuii*,[1,2] for their unique properties. Nanoalgosomes have demonstrated several advantages, including good tolerance in living organisms and effective internalization into the intestinal cells of *C. elegans*.[3] Taking advantage of these features, nanoalgosomes have been loaded with neuroprotective compounds capable of mitigating Spinal Muscular Atrophy (SMA) related neurodegeneration in *C. elegans*. SMA is genetic disease causing motoneuron loss and severe dysfunctions. Neuroprotective compounds have been effectively encapsulated into EVs using saponin treatment as a loading technique and subsequently purified using size exclusion chromatography. The nanosystems were characterized by different biophysical and biochemical techniques, including fluorescence to quantify the loaded cargo.
In vivo testing on a *C. elegans* SMA model has revealed that neuroprotective compounds encapsulated within nanoalgosomes exhibit greater efficacy compared to free drugs with a drastic reduction of active dose. This emphasizes the enhancement of neuroprotective drug efficacy through nanoalgosome encapsulation.

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OPTIMIZING HYDROGEN PRODUCTION IN MICOALGAE

Simone Botticelli,[1,2] Giovanni La Penna,[2,3] Velia Minicozzi,[1,2] Francesco Stellato,[1,2] Silvia Morante,[1,2] Giancarlo Rossi,[4] Silvia Pizzanelli,[3] Fabrizio Machetti,[3] and Cecilia Faraloni[5]

[1] Department of physics, University of Roma Tor Vergata, Roma, Italy
[2] National institute of nuclear physics, Section of Roma Tor Vergata, Italy
[3] National research council, Institute of chemistry of organometallic compounds, Firenze & Pisa, Italy
[4] Museo Storico della Fisica e Centro Studi e Ricerche E. Fermi, Roma, Italy
[5] National research council, Institute of bioeconomy, Firenze, Italy
giovanni.lapenna@cnr.it

Microalgae produce, in particular conditions, pure hydrogen gas from water using visible light as energy source. The genus *Chlorella* showed a good hydrogen yield, easy growth conditions, and a moderate resistance to oxygen levels when hydrogen is produced.[1] Despite the energy to produce hydrogen is taken by photosynthesis (reduced ferredoxin), when photosynthesis becomes efficient and oxygen production increases, the hydrogen production is switched off by the achieved level of oxygen. The oxygen sensitivity of the essential enzyme involved in hydrogen production, hydrogenase, can be optimized by genetic engineering, thus allowing an efficient cycling between light and dark phases and achieving a sustainable hydrogen production and cell growth.

The structure of hydrogenase of *Chlorella vulgaris*, an algal strain promising in biotechnology, is not known. A draft protein structure was predicted by AlphaFold, a deep-learning procedure recently developed. We added iron-sulfur components to the predicted structure achieving a novel fully functional structural model for the enzyme by combining enhanced molecular dynamics methods and deep-learning proposals.[2] The hydrogenase model explains the larger oxygen resistance of *C. vulgaris* compared to other microalgae.

To understand the peculiar iron-sulfur biogenesis when hydrogen production is maximal, we interpreted for the first time the electron spin resonance spectra of whole *C. vulgaris* cells in different metabolic states. The results provide a magnetic marker of hydrogenase-ready iron-sulfur clusters emerging in the hydrogen-active cell state.

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A MULTISCALE APPROACH TO STUDY ANGIOTENSIN-CONVERTING ENZYME 2 (ACE2) AND ITS PEPTIDE INHIBITOR DX600

Alessandro Mossa,[1] Valentina Tozzini,[2] and Giorgia Brancolini[1]

[1]Istituto Nanoscienze - CNR, Via G. Campi 213/A, 41125 Modena, Italy
[2]NEST, Scuola Normale Superiore and Istituto Nanoscienze - CNR, Piazza S. Silvestro 12, 56127 Pisa, Italy
alessandro.mossa@nano.cnr.it

Most of the computational techniques employed in drug design have been originally introduced and optimized for the case of a small molecule interacting with a protein. As pharmaceutical research is gradually shifting its interest towards the use of short peptides instead of small molecules, the need to modify old methods or devise new ones is becoming more urgent. In this contribution we show how a multiscale approach combining atomistic and coarse grained simulations is able to clarify the (as yet unresolved) molecular details of the interaction between ACE2,[1,2] the membrane protein acting as a receptor for SARS-CoV-2 spike,[3] and the peptide inhibitor of its enzymatic action called DX600.[4] Apart from the intrinsic applicative interest of this complex, which is strong due to the roles played by ACE2 both in COVID-19 infection and in the function of the renin-angiotensin-aldosterone system,[5] the strategy we adopted lends itself to be easily accommodated to similar protein-peptide interactions.

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DESIGN OF PEPTIDE INHIBITOS OF PROTEIN-PROTEIN INTERACTIONS TARGETED TO SRC HOMOLOGY 2 DOMAINS: A COMBINED SIMULATIVE AND SPECTROSCOPIC APPROACH

C. Innamorati[1], C. Storti[1], P. Calligari[1], S. Bobone[1], C. Fulci[1], C. Cocca[1], C. Peggion[2], B. Biondi[2], G. Bocchinfuso[1] and L. Stella[1]

[1]Department of Chemical Sciences and Technologies, University of Rome "Tor Vergata", Rome, 00133, Italy
2Department of Chemical Sciences, University of Padua, Padua, 35131, Italy
*stella@stc.uniroma2.it

Src homology 2 (SH2) domains are modules of about 100 amino acids, which mediate protein-protein interactions by recognizing sequences comprising a phosphorylated tyrosine residue[1]. Due to their role in numerous signalling pathways, they have garnered significant attention as potential targets for pharmaceutical intervention. The SHP2 phosphatase comprises two SH2 domains and it is of particular interest due to its role in regulating various cellular processes[2,3]. In the last years, we have developed and patented potent oligopeptides targeting the N-terminal SH2 domain of SHP2[4]. We are currently combining in silico studies and spectroscopic experiments to optimize these molecules and bring them closer to clinical application. Potential of mean force calculations allowed us to screen several possible sequence modifications, including the insertion of non-natural amino acids. The affinity of these peptides to the target domain was tested by fluorescence anisotropy binding assays. A good correlation was observed between the calculated and experimentally determined binding standard free energies, and a sequence with improved affinity was identified, reaching a single digit nM dissociation constant. The selectivity of the inhibitors was characterized by combining high throughput experiments on solid supported arrays of human SH2 domains, and homogeneous binding assays in solution. These studies showed that fluorescent labeling can significantly perturb the measured binding affinities. Importantly, the specific effects depend on the fluorophore and on the SH2 domain being considered. Fortunately, competition displacement assays can be used to measure the real binding affinity of unlabeled peptides. When the fluorophores' effects were removed, the selectivity of our peptides for their intended target resulted to be in the order of 10^3-10^4. Finally, we applied our approach to design inhibitors targeted to the other SH2 domain of SHP2, termed C-SH2. Analyzing the sequences of natural binders, peptide libraries studies, X-ray structures of peptide/domain complexes, and MD simulations, we identified the structural determinants of sequences specifically recognized by the C-SH2 domain. Starting from a natural sequence, our binding assays demonstrated that a minimal peptide of just eight residues is sufficient for an affinity corresponding to a low nM dissociation constant. Overall, the specific test case of SHP2 shows that the combined use of computational and spectroscopic methods can lead to the development of highly potent and selective oligopeptide inhibitors targeted to SH2 domains.

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AN MD SIMULATION-ENHANCED MACHINE LEARNING VIRTUAL SCREENING FOR ACCUMULATION OF ANTIMICROBIALS

Stefan Milenkovic and Matteo Ceccarelli**

*Physics Department, University of Cagliari, Italy

The rise of antibiotic resistance poses a critical challenge to global healthcare, demanding the discovery of novel antibiotics. However, this endeavor is hindered by several bottlenecks, particularly for Gram-negative bacteria, where assessing the permeability of small molecules through outer membranes lacks a systematic approach. An efficient screening of vast libraries of drug-like compounds is essential for antimicrobial discovery. In this work, we introduce a machine learning (ML) model for predicting the permeability and accumulation of small molecules in Enterobacteriaceae. Our innovative approach incorporates statistical properties of selected molecular descriptors derived from Molecular Dynamics (MD) simulations. Traditionally, models rely on average values, assuming a normal distribution, potentially overlooking vital information. Small molecules, however, exhibit diverse conformational states with varying frequencies, leading to non-normal descriptor distributions. By integrating higher-order statistical moments derived from MD simulations, we enhance existing models. Our work quantifies the improvement achieved through this integration and proposes a virtual screening workflow. This workflow allows for the rapid identification of small molecules with optimal permeability and accumulation, thereby advancing the discovery of antimicrobial agents.

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MAGNETIC FIELD OF CARDIAC CELL CULTURES: A THEORETICAL-COMPUTATIONAL STUDY

Martina Nicoletti,[1,2] Alessandro Loppini,[3] Alessio Gizzi,[1] Christian Cherubini,[4] Letizia Chiodo[1] , and Simonetta Filippi[1]

[1]Department of Engineering, Università Campus Bio-Medico di Roma, Via Alvaro del Portillo 21,0.00128 Roma, Italy
[2]Center for Life Nano and Neuro Science, Istituto Italiano di Tecnologia, Viale Regina Elena 291,00161 Roma, Italy
[3]Department of Medicine and Surgery, Università Campus Bio-Medico di Roma, Via Alvaro del Portillo 21,0.00128 Roma, Italy [4]Department of Science and Technology for Sustainable Development and One Health, Università Campus Bio-Medico di Roma, Via Alvaro del Portillo, Rome, 21-I-00128, Italy
m.nicoletti@unicampus.it

Gaining a comprehensive understanding of the origins of cardiac instabilities and the progression of arrhythmias is of critical importance for clinical applications. While the transition from regular rhythms to irregular activation has been widely explored using standard indicators based on voltage and calcium signals, the underlying thermo-magnetic effects are still poorly explored. However, they could offer new insights into the emergence of cardiac instabilities and the onset and development of arrhythmias. In this contribution, we present a first-of-its-kind theoretical framework comprising cardiac cell electrophysiology and associated electro-magnetic field generation. By coupling phenomenological models of cardiac electrical activity with reaction-diffusion equations and Maxwell equations, we perform a finite-element-based numerical investigation of the magnetic field produced by cardiac cell activity. In particular, by varying the thermal state of the tissue and conducting magnetic field analyses, we examine the thermo-magnetic activity of the tissue for common stimulation patterns, including planar waves, target patterns, and spiral waves (prototype of cardiac arrhythmias). We devise a tool for investigating cardiac activity from a new perspective, which could improve our understanding of cardiac cell function in health and disease.

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Selezionati per

POSTER in **BIOFISICA**

A PHOTOACTIVE SUPRAMOLECULAR COMPLEX TARGETING PD-L1 REVEALS A WEAK CORRELATION BETWEEN PHOTOACTIVATION EFFICIENCY AND RECEPTOR EXPRESSION LEVELS IN NON-SMALL-CELL LUNG CANCER TUMOR MODELS

Manuela Maria Alampi [1] , Pietro Delcanale [1], Andrea Mussini [1] , Claudia Fumarola [2] , Maricla Galetti [3] , Pier Giorgio Petronini [2] , Cristiano Viappiani [1] and Stefania Abbruzzetti [1]

[1]Department of Mathematical, Physical and Computer Sciences, University of Parma, 43124 Parma, Italy
[2]Department of Medicine and Surgery, University of Parma, 43125 Parma, Italy
[3]Department of Occupational and Environmental Medicine, Epidemiology and Hygiene, INAIL-Italian
Manuelamaria.alampi@unipr.it

The use of immunoconjugates provided with photosensitizing and targeting properties for cancer treatment is a promising strategy that combines photodynamic therapy (PDT) and immunotherapy[1]. For both these applications, the superficial density of receptors on tumor cells is considered a key factor in predicting the outcome of the treatment[2]. In this work, we developed a photoactive supramolecular system able to selectively target the membrane receptor programmed death-ligand 1 (PD-L1) to investigate whether the expression level of PD-L1 can determine a priori the efficacy of the photoimmunotherapy (PIT) treatment. At this purpose, either the photosensitizer eosin or the fluorescent probe Alexa647 was covalently conjugated to the clinical grade PD-L1-binding monoclonal antibody Atezolizumab. First, we used an anti-PD-L1 monoclonal antibody labeled with Alexa647 to quantify PD-L1 receptors in two human non-small-cell lung cancer lines (H322 and A549) expressing PD-L1 to a different level by direct stochastic optical reconstruction microscopy (dSTORM)[3]. dSTORM was also used to assess the binding between Atezolizumab and its target PD- L1 on the selected cell lines. Finally, we proved that the photo-immunoconjugate is suitable for PDT treatment. However, our results show no correlation between the efficacy of PIT treatment and the density of PD-L1 receptors on the cell surface, suggesting that a higher expression of receptors does not necessarily determine a better treatment outcome and that the treatment can be effective also in the case of low-PD-L1-expressing cells.

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GREED AND ALTRUISM: HOW QUORUM SENSING MEDIATES BETWEEN THESE TWO BEHAVIORS

Eleonora Alfinito,[1] *Matteo Beccaria*[1,2,3]

[1]Dipartimento di Matematica e Fisica 'Ennio De Giorgi' -Università del Salento, via Arnesano, 73100, Lecce, Italy
2Istituto Nazionale di Fisica Nucleare - sezione di Lecce, Via Arnesano, I-73100 Lecce, Italy
3National Biodiversity Future Center, Palermo 90133, Italy.
eleonora.alfinito@unisalento.it
matteo.beccaria@unisalento.it

Greed and altruism are two sides of the same coin, and represent opposite antagonistic mechanisms that may lead communities of social beings to extinction. Bacterial strains consisting of unconditional cooperators (UC) or defectors (DC) in relation to the production of public goods, have been analyzed under different experimental conditions[1] and both die out quickly. UC are characterized by a low growth efficiency and become extinct due to aging even in presence of leftover resources. DC are greedy, grow fast, and rapidly run out of food. On the other hand, well-mixed UC and DC populations survive longer. Quorum sensing (QS)[2] is the ability of bacteria to cooperate when a density threshold is reached, and was proposed as the *deus-ex-machina* that presides over this phenomenon [1]. In previous works [3,4] we proposed a model describing the bacterial colony formation and its related bioluminescence. In this description, both phenomena are mediated by a long-range interaction which implements QS (QSa).
Here, modeling is improved to account for the presence of two different types of cooperators. By appropriately modifying the parameters of the model, it is possible to reproduce UC or DC behavior.
We analyze by numerical simulations the evolution of a mixed state of both these strains and examine the role of QSa in its stability over time. We show that it effectively acts as a regulator by mediating resource distribution based on UC, DC densities with the effect of increasing the typical survival time compared to that of pure states.

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THE IMPACT OF EXTERNAL MECHANICAL CUES ON THE CELLULAR LOCALIZATION AND THE ACTIVATION OF PIEZO 2 CHANNELS.

Giorgia Demontis[1,2], Miriam De Sarlo[3], Paul Heppenstall[4], Fernanda De Castro Reis[4], Marco Cecchini[3], Ilaria Tonazzini[3], Laura Andolfi[1]

1 Istituto Officina dei Materiali-Consiglio Nazionale delle Ricerche (IOM-CNR), Basovizza, Trieste (Italy)
2 University of Trieste, 34100 Trieste (Italy)
3 Istituto Nanoscienze-Consiglio Nazionale delle Ricerche (CNRnano) @NEST, piazza San Silvestro, Pisa (Italy).
4 Scuola Superiore Studi Avanzati (SISSA) Via Bonomea, Trieste (Italy)

Mechanotransduction largely relies on ion channels that respond to mechanical forces. Piezo1 and Piezo2 proteins were discovered to be the biggest non-selective mechanosensitive cation channels in mammalian.[1] Piezo 2 has a specific sensory function and is primarily found in merkel cells and dorsal root ganglion (DRG) neurons. Piezo 2 channels are the primary transducers of the mechanical stimulation for the sense of touch, and they are found in the afferent fibers.[2] Little is known about the Piezo 2 channel polarization in these sensory cells as well as the Piezo 2 activation mechanism. To investigate whether mechanical or topographical cues prompt the localization of Piezo 2 within specific cellular regions, we fabricated micro-grooved substrates for culturing HEK-Piezo 2 green- lantern transfected cells as an overexpression system, or dorsal root ganglion (DRG) neurons naturally expressing Piezo 2. Furthermore, to investigate the forces activating the Piezo 2 channels, we employed Atomic Force Microscopy (AFM) and Fluidic Force Microscopy (FluidFM) combined with calcium imaging. Both techniques allow for the application of a local stimulus to cellular regions with controlled force intensity and direction, including positive and negative pressure. Our results demonstrate that micro-grooved substrates, with a period of 2 μm and 20 μm (period= ridge + groove width), efficiently orient axonal growth of DRG. On the same grating the HEK-Piezo 2 green-lantern transfected cells exhibit Piezo 2 channels primarily situated at the cell periphery, aligning predominantly with the patterned lines. In contrast, on flat substrates, Piezo 2 appears uniformly distributed throughout the cells. Indentation by a standard AFM probe of HEK-Piezo 2 green-lantern transfected cells cultured on flat substrates, reveals that a greater proportion of transfected cells exhibit calcium influx at 5 nN compared to control cells. The integration of microfabrication methodologies with techniques capable of precisely applying controlled mechanical stimuli is expected to significantly enhance our understanding of mechanotransduction mechanisms in DRG. This advancement carries the potential to shed light on issues associated with pathologies such as allodynia and chronic pain.

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SUPER-RESOLUTION FLUORESCENCE MICROSCOPY COUPLED WITH A DOUGHNUT-SHAPED TWO-PHOTON EXCITATION BEAM TO STUDY DNA ORGANIZATION IN THE CELL NUCLEUS

Elena Angeli[1], Elena Gatta[1], Virginia Bazzurro[1], Colin Sheppard[2,3], Paolo Bianchini[1,3], Alberto Diaspro[1,3,4]

[1]SEELIFE, Department of Physics, University of Genoa, Italy
[2]Wollongong University, Australia.
[3]Nanoscopy, CHT – Istituto Italiano Tecnologia, Genoa, Italy
[4] Institute of Biophysics, CNR, Genova, Italy
Elena.angeli@unige.it

Here, we report how, by using the very same doughnut beam provided in a commercial STED (stimulated emission depletion) microscope, we succeeded in combining super-resolution imaging with two-photon excitation of UV-absorbent fluorophores such as DAPI and Hoechst. For this purpose, we used a high-power pulsed doughnut-shaped laser provided by the Stellaris 8-TAU-STED microscope (Leica Microsystems), which is conventionally used for depletion in STED imaging; this laser operates at 775 nm with pulses 550-750 ps long modulated at 80 MHz.
We studied how different parameters, such as the power of the doughnut beam and scan speed, affect the emission signal properties like spatial resolution, optical sectioning, etc. Moreover, we compared the results with those obtained by single-photon confocal imaging using the supercontinuum white light laser source provided on the Stellaris 8 microscope. We took advantage of this approach to study DNA organization in the cell nucleus by using DAPI and Hoechst to stain DNA.

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UNVEILING PROTEIN BEHAVIOR IN CONFINED ENVIRONMENTS: EXPLORING PH-INDUCED AGGREGATION DYNAMICS

Sara Anselmo, Giuseppe Sancataldo, Giuseppe Arrabito, Bruno Pignataro and Valeria Vetri

Dipartimento di Fisica e Chimica-Emilio Segrè, Università degli studi di Palermo, Viale delle Scienze, 90128 Palermo, Italy
sara.anselmo@unipa.it

Understanding protein supramolecular assembly is fundamental for multiple scientific domains, impacting human health, biotechnology, and material science alike. Its involvement in various neurodegenerative diseases underscores its critical relevance, while its role in cellular processes offers insights into fundamental biological functions. Additionally, protein aggregates can serve as building blocks for the design and fabrication of innovative biomaterials with unique properties. Given that biological reactions predominantly occur within enclosed microenvironments such as cells and organelles, the study of molecular reactivity and behaviour due to spatial constraints is a topic of paramount interest. Confined environments can in fact significantly affect protein structure, folding, conformational stability and aggregation dynamics. In the context of the project "Sicilian Micro and Nano Technology Research and Innovation Center (SAMOTHRACE)", our focus is on the investigation of the behaviour of bovine serum albumin (BSA) within confined spaces, particularly in response to pH fluctuations induced by the release of H+ ions in solution following the dissociation of nitrobenzaldehyde under UV irradiation in restricted sample volumes which mimic cellular environments. To produce aqueous compartments, we use inkjet printing to generate arrays of droplets containing proteins, reactive molecules, and polymers, ranging in volume from nanoliters to femtoliters. These droplets are introduced into mineral oil to replicate the basic characteristics of "cell- like" compartments on a chip platform. Raster image correlation spectroscopy (RICS) is then employed for real-time analysis of molecular diffusion within these compartments, adapting to changing environmental conditions. This enables the precise monitoring of protein aggregation processes.

SIMULATION BASED ANALYSIS OF TYPE-0 NON-DEGENERATE PPKTP CRYSTAL FOR SPDC PROCESS

Gaytri Arya,[1] Paolo Bianchini,[1,2] and Alberto Diaspro,[1,2]

[1]Nanoscopy, Istituto Italiano Tecnologia, Genoa, Italy
[2]DIFILAB Department of Physics, University of Genoa, Genoa, Italy
gaytri.arya@iit.it

Spontaneous Parametric Down-Conversion is a key phenomenon in numerous quantum technologies like quantum imaging for its ability to produce entangled photon pairs.[1] Quantum ghost imaging, quantum-enhanced imaging and quantum imaging with undetected photons have merits of reconstructing biological samples with high resolution in presence of significant background noise, surpassing classical limits in image sensitivity and detection in visible region when sample interaction lies in infrared region.[2,3] Leveraging SPDC, this work focuses on the simulation-based characterization of periodically polled Potassium Titanyl Phosphate (PPKTP) type-0 non-degenerate crystal in collinear configuration. PPKTP, a ferroelectric crystal with inverse domains formed during poling, is well-suited for SPDC due to its high photon yield resulting from efficient non-linear optical properties.[4,5] The study of variation of signal and idler wavelengths with temperature, effect of beam-waist, spectral density of signal-idler photons and joint photon spectrum has been discussed for the customized crystal (1 x 2 x 2 mm^3). Our findings provide crucial insights into optimizing the efficiency and spectral properties essential for quantum imaging applications, particularly in the realm of biophysics. By elucidating the behaviour of these crystals under varying conditions, our study offers valuable guidance for designing advanced imaging techniques that can revolutionize biological imaging and analysis at quantum level. We acknowledge the funding for this project received from NPRR MUR project PE00000023 – NQSTI .

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EXPLORING CHROMATIN AND EPIGENOME ALTERATIONS IN NEUROBLASTOMA TRANSFORMATION

Francesca Baldini,[1] Lama Zeaiter,[1,2] Lisa Cuneo,[1,3$] Paolo Bianchini,[1] Laura Vergani,[2] Aldo Pagano,[4,5] and Alberto Diaspro[1,3]

[1]Nanoscopy, Istituto Italiano Tecnologia, Genoa, Italy
[2]DISTAV, Department for the Earth, Environment and Life Sciences, University of Genoa, Genoa, Italy
[3]DIFILAB Department of Physics, University of Genoa, Genoa, Italy
[4]IRCCS Ospedale Policlinico San Martino, Genoa, Italy
[5]DIMES, Department of Experimental Medicine, University of Genoa, Genoa, Italy
[$] Current address: CSML & MMS, Istituto Italiano di Tecnologia, Via Enrico Melen 83, Genoa, Italy

Neuroblastoma (NB) is the most common extracranial solid tumor in childhood. This cancer shows remarkable heterogeneity in its clinical phenotypes and distinct evolutionary patterns, and, despite the advancements, the molecular mechanisms are still partially unknown. However, the impairment of the "epigenetic machinery" and the consequent chromatin reorganization might play an important role.[2] Our aim was to delineate alterations in nuclear and chromatin architecture associated with NB retro-transformation. To achieve this, we employed the SKNBE2 neuroblastoma cell line, engineered to overexpress the non-coding RNA NDM29, inducing transformation from highly malignant NB cells (Mock) to less malignant, neuron-like cells (S1.1).[2] Using confocal microscopy, we examined potential alterations in nuclear morphology resulting from NDM29 overexpression.[3] Also, we employed stimulation emission depletion (STED) imaging[4] coupled with histone H3 labeling to probe changes in various epigenetic domains. We assessed the overall DNA methylation through enzyme- linked immunoassay and the mRNA expression of key epigenetic modifying enzymes and a panel of NF-κB-regulated genes using quantitative PCR and a cDNA-plate array, respectively. Our findings unveiled elongated nuclei with reduced volume in S1.1 cells, alongside remodeling of H3 epigenetics and increased DNA methylation. Functionally, these cells exhibited enhanced expression of the acetyltransferase KAT2A and reduced expression of the methyltransferase EHMT2, along with downregulation of most NF-κB-regulated genes. These observations underscore a reorganization of nuclear structure and epigenetic landscapes during NMD29-induced NB retro-transformation, impacting gene expression. These insights enhance our understanding of the molecular mechanisms underpinning neuroblastoma heterogeneity and malignancy, offering promising avenues for prognostic and therapeutic interventions at a nuclear level.

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FROM PROTEIN-LIGAND INTERACTION TO PROTEIN REGULATION: INSIGHTS FROM MOLECULAR DYNAMICS SIMULATIONS

Alessio Bartocci[1,2] *Costanza Paternoster*,[1,2] *Giovanni Novi Inverardi*,[1,2], *Francesco Carnovale*[1,2] *and Gianluca Lattanzi*[1,2]

[1]Department of Physics - University of Trento, Via Sommarive 14, 38123 Trento, Italy
[2]INFN-TIFPA, Trento Institute for Fundamental Physics and Applications, Via Sommarive 14, 38123 Trento, Italy
alessio.bartocci@unitn.it

Bindings of small ligands to proteins are ruled by interactions between the ligand and residues in the protein's binding site, which can be located in a small binding pocket or on the protein surface. Such interactions can directly modulate protein functions and conformations, with possible implications ranging from biophysical processes, such as protein co-crystallization[1-3], to the understanding of molecular mechanisms behind drug binding[4] (selective drug design). Computational ways to investigate such systems are molecular dynamics simulations (MDs) which, spanning from atomistic to coarse-grained resolution, can give insights into the mechanisms, kinetics, and thermodynamics underpinning the protein-ligand complex formation. Here, it will be discussed how supramolecular complexes, formed by proteins interacting with small molecules, can be described by MDs approaches, proposing characteristic fingerprints and key patterns for the interactions.

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LAB-ON-CHIP FOR SUSTAINABLE OLIVE VALUE CHAIN [LOVE]: DEVELOPMENT OF A LAB-ON-CHIP FOR DETECTING POLYPHENOLIC COMPOUNDS IN OLIVE AND ITS DERIVATIVES

Antonella Battisti,[1] Giorgia Tori,[2] Mariacristina Gagliardi,[1] Marco Cecchini,[1] and Chiara Sanmartin[3]*

[1]NEST, Istituto Nanoscienze – CNR, Piazza S. Silvestro 12, 56127 Pisa, Italy
[2]NEST, Scuola Normale Superiore, Piazza S. Silvestro 12, 56127 Pisa, Italy
[3]Dipartimento di Scienze Agrarie, Alimentari e Agro-Ambientali, Università di Pisa, Via del Borghetto 80, 56124 Pisa, Italy
*antonella.battisti@nano.cnr.it

Polyphenols have gathered increasing attention during the last decades because of their potential health benefits as antioxidant. Epidemiological studies and systematic reviews reveal their role in the protection from pathologies such as cancers, diabetes, osteoporosis, cardiovascular and neurodegenerative diseases.[1] Polyphenols often play a role in the defense of plants, where they are produced as secondary metabolites, against ultraviolet radiation or aggression by pathogens. They are key compounds in olives and their concentration can be used to determine the ripening degree.[2] An optimal ripening degree corresponds to high quality fruits that can lead to high quality olive derivatives. In contrast with a current frame where samples are collected, road transported to the analytical lab, analyzed with traditional techniques for a few parameters and results are sent back after some hours or days, the LOVE project proposes a simple, cost-effective, and time-efficient strategy to assess the phenolic content in olives. This is achieved thanks to an acoustic sensor with an optimized surface for the rapid detection of polyphenols in olive extracts, in analogy to what has been obtained for wine products,[3] especially for the determination of olive ripeness. The expected results will show that the use of this platform could be a viable approach to perform a fast screening of olive quality, opening the way for devices able to provide direct *in situ* real-time assessments of olives ripening for a more efficient farming system and higher-quality production.

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FLUORESCENCE LIFETIME COMBINED WITH STIMULATED EMISSION DEPLETION MICROSCOPY FOR INVESTIGATING $GABA_A$ RECEPTOR SUBUNITS' RESPONSE TO ANTISECRETORY FACTOR

Virginia Bazzurro[1], Elena Gatta[1], Elena Angeli[1], Aroldo Cupello1, Stefan Lange[2,3], Eva Jennische[4], Mauro Robello[1], Alberto Diaspro[1,5]

[1]SEELIFE and DIFILAB, Department of Physics, University of Genoa, Genoa, Italy.
[2]Department of Infectious Diseases, Institute of Biomedicine, University of Gothenburg, Gothenburg, Sweden.
[3]Region Västra Götaland, Department of Clinical Microbiology, Sahlgrenska University Hospital, Gothenburg, Sweden.
[4]Department of Medical Biochemistry and Cell Biology, Institute of Biomedicine, Sahlgrenska Academy, University of Gothenburg, Gothenburg, Sweden.
[5]Nanoscopy, CHT Erzelli, Istituto Italiano di Tecnologia, Genoa, Italy.
Virginia.bazzurro@unige.itt

The antisecretory factor (AF) is a protein that can reduce intestinal hypersecretion and various inflammation disorders, but its mechanism of action is still unknown.
We previously studied the role of AF on $GABA_A$ receptors in cerebellar granule cells, taking advantage of patch-clamp in whole-cell configuration; in this work, we performed immunofluorescence experiments by staining α_1 and α_6 subunits before and after AF-16 administration in order to investigate the interaction of AF with $GABA_A$ receptors.
We used STED microscopy combined with fluorescence lifetime microscopy to create super-resolved 3D maps of receptorial subunits distributed on the neurons and we analyzed super-resolved images comparing pre- and post-treatment maps and observed differences on how different subunit populations respond to AF treatment. We critically compared these new experimental results with our previous electrophysiological data to shed light on the mechanisms of action of AF protein on $GABA_A$ receptor subpopulations.

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SENSING NITRIC OXIDE WITH A FLUORESCENT PROTEIN

Lucia Bellanova,[1] Keren Morreale,[1] Arne Franzen,[2] Beatrix Santiago-Schübel,[3] Stefano Bruno,[4] Antonio Scarano, [4] Stefania Abbruzzetti,[1] Pietro Delcanale,[1] Cristiano Viappiani,[1] Thomas Gensch[2]

[1]Dipartimento di Scienze Matematiche, Fisiche e Informatiche, Università di Parma, Parco Area delle Scienze 7A, 43124 Parma, Italy

[2]Institute of Biological Information Processing (IBI-1: Molecular and Cellular Physiology), Forschungszentrum Jülich, Leo-Brandt-Straße, D-52428 Jülich, Germany

[3]Zentralinstitut für Engineering, Elektronik und Analytik, ZEA-3, Forschungszentrum Jülich, 52425 Jülich, Germany

[4]Dipartimento di Scienze degli Alimenti e del Farmaco, Università di Parma, Parco Area delle Scienze 27A, 43124 Parma, Italy

lucia.bellanova@unipr.it

Real-time quantitative visualization of nitric oxide (NO) concentration at the single cell level is fundamental to achieve a direct and accurate determination of NO dynamics. To this aim, development of genetically encoded fluorescent sensors (GES) is of fundamental relevance. The field is still poorly explored and only a few GES have been so far developed and proposed for monitoring intracellular NO levels [1].

The recent finding that the blue-emitting fluorescent protein mTagBFP2 is sensitive to NO in the micromolar range suggested new development strategies [2]. Protein mutants and mass spectrometry demonstrated that S-nitrosylation of Cys residues is at the basis of the observed reduction in emission intensity and lifetime in response to NO exposure. The potential of this GES for monitoring intracellular NO was shown on HeLa cells transiently expressing mTagBFP2.

In this work, we present a more accurate study on the dynamics of S-nitrosylation of Cys residues in mTagBFP2. S-nitrosylation of mTagBFP2 was induced by the NO donor MAHMA nonoate and the changes in fluorescence intensity and lifetime were monitored as a function of time. From the overall change, it was possible to obtain a better estimate of the K_d for the reaction.

We have also explored the response of mTagBFP2 to the NO donors S-nitrosoglutathione (GSNO) and S-nitroso-N-acetylpenicillamine (SNAP). Experiments on mTagBFP2 mutants containing a single Cys residue show that two of the three Cys residues in mTagBFP2 are responsible for the changes in fluorescence emission. Further experiments on bacterial and mammalian cells expressing mTagBFP2 and its mutants, are underway.

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INVESTIGATING TMEM16E MUTATIONS IMPLICATED IN MUSCULAR DYSTROPHY AND GDD SKELETAL DYSPLASIA

Anna Boccaccio[1,2], Cristiana Picco[1], Joachim Scholz-Starke[1]

1 Institute of Biophysics, National Research Council, Genova, Italy
2 Raise Ecosystem Genova, Italy anna.boccaccio@ibf.cnr.it

TMEM16E/ANO5 is a membrane protein that is highly expressed in skeletal muscle, cardiac muscle, chondrocytes, and osteoblasts. Mutations in the human *TMEM16E/ANO5* gene are causative for gnathodiaphyseal dysplasia (GDD), a rare bone malformation and fragility disorder, and for two types of muscular dystrophy (MD, limb-girdle muscular dystrophy type 12, LGMDR12 and Miyoshi muscular dystrophy type 3). We demonstrated that TMEM16E/ ANO5 is a Ca^{2+}- activated phospholipid scramblase (lipid translocation) showing nonselective ion translocation with slow time-dependent activation at highly depolarized membrane potentials (Di Zanni et al 2018, 2020).
Understanding the molecular mechanisms underlying TMEM16E-related pathologies is essential for the correct disease identification, development of targeted therapeutic strategies aimed at ameliorating the symptoms and improving the quality of life for affected individuals.
Using established HEK293-based functional assays, we investigated the effects of MD-related and GDD-related amino acid exchanges on TMEM16E/ANO5 function in the same expression system. Our results collectively indicate that GDD mutations are associated to a gain-of-function phenotype on the level of protein function, confirming conjectures made on the basis of inheritance mode. MD mutations associated to a recessive inheritance instead lead to loss-of-function.
Contrary to this clear-cut dichotomy however, we identified a novel, *de novo* heterozygous *ANO5* variant in a patient with a history of recurrent rhabdomyolysis, elevated CK, and leg muscle pain. Our functional studies identified a gain-of-function effect caused by the variant, supporting a new *ANO5* autosomal-dominant muscular condition.

TOWARDS THE DESIGN OF FUSOGENIC NANOPARTICLES: PHYSICAL DETERMINANTS OF NANOPARTICLE-INDUCED STALK FORMATION

Davide Bochicchio,[1] Giorgia Brosio,[1] Paolo Volpe,[1] Giulia Rossi[1]

[1]Department of Physics, University of Genova, Via Dodecaneso 33, 16146, Genova, Italy
davide.bochicchio@unige.it

Membrane fusion is indispensable for various biological functions, such as neurotransmitter release and intracellular trafficking. *In vivo*, this intricate, multi-step process is regulated by specialized proteins, which cooperate with specific lipid compositions and ionic environments. Synthetic approaches for controlled membrane fusion have been recently endeavored and will be crucial for next-generation biomedical applications.[1] However, controlling fusion rates via synthetic agents is challenging and requires a profound comprehension of the molecular mechanisms involved in the distinct fusion steps. In this context, molecular simulations are a valuable tool for investigating the interaction between the fusogenic agents and the lipid membranes with spatial and temporal resolutions not reachable in the experiments.

Recently, we focused on understanding what determines the efficiency of functionalized gold nanoparticles (Au NPs) in inducing stalk formation (the first fundamental step in the fusion path), employing coarse-grained molecular dynamics complemented with enhanced sampling. In a first study conducted in parallel with experimental collaborators, we demonstrated cooperative effects between the membrane lipid composition and the Au NPs, showing that cholesterol-containing membranes are especially prone to undergo NP-mediated fusion.[2] Then, we developed an enhanced sampling procedure to calculate the free energy profile of NP-induced stalk formation and used it to reveal the effect of the nanoparticle core size.[3] In particular, we showed that smaller nanoparticles are more efficient in inducing the stalk between nearby flat membranes. More recently, we investigated the interplay between the NP core size and the membrane curvature, allowing for interpreting experimental results on liposomes of different sizes.

Our studies provide general insights into the molecular mechanisms underlying NP-mediated membrane fusion, suggesting design principles for synthetic, fusogenic NPs.

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TOWARDS PERSONALIZED MEDICINE: INVESTIGATING PARKINSON'S DISEASE BY PATIENT-DERIVED MIDBRAIN ORGANOIDS.

Diego Lopez[1,2], Saralea Marino[1,2], Micaela Pannella[3], Emanuele Frattini[4], Ilaria Trezzi[4], Claudia Saraiva[5], Alessio Di Fonzo[4], Jens Schwamborn[5] and Mario Bortolozzi[1,2]

[1]Department of Physics and Astronomy "G. Galilei", University of Padua, Padova, Italy
[2] Veneto Institute of Molecular Medicine (VIMM), Via Orus 2, Padova, Italy
[3]IRCSS Istituto Ortopedico Rizzoli, Bologna, Italy
[4] Neurology Unit, IRCCS Foundation; Granda Ospedale Maggiore Policlinico, Via Francesco Sforza 35, 20122, Milan, Italy
[5]Luxembourg Centre for Systems Biomedicine (LCSB), University of Luxembourg, Luxembourg

One of the most exciting advancements in stem cell research of the last few years has been the development of human brain organoids. This *in vitro* system consists of multiple cell types that can self-organize in three-dimensions representing a brain region able to recapitulate physiological and pathological relevant aspects. Compared to animal models, patient-derived organoids provide emerging prospects for testing new drugs and developing precision medicine. Human midbrain organoids (hMBOs) can mimic the *sunstantia nigra*, the brain region that degenerates in Parkinson's disease (PD), including the complex interaction of dopaminergic neurons with other types of neurons and glial cells. In this work, we characterized hMBOs derived from healthy subjects and PD patients carrying monogenic mutations of *RHOT1* and *PARK2* genes identified to cause Parkinson's disease (PD) in a highly penetrant manner.
In order to address the complexity of hMBOs, we combined patch-clamp, multielectrode arrays (MEA) and two-photon microscopy. Monitoring the electrical activity and Ca^{2+} dynamics at both the single cell and network level permitted us to gather extended information about the functionality of healthy and PD-linked organoids. The experiments unveiled the emergence of a synchronous neuronal activity in hMBOs at 60-90 days as previously reported in other types of cerebral organoids[1,2]. We demonstrated that the dopaminergic population acts as a pacemaker of the global network activity, similarly to what observed in the human brain. Finally, we tested a drug with the potential to revert or mitigate the neurodegeneration caused by a class of PD mutations linked to the *PARK2* gene.

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SHEDDING LIGHT ON BETA-AMYLOID AGGREGATION BY MEANS OF ATOMISTIC AND COARSE-GRAINED MOLECULAR DYNAMICS

*Irene Cadenelli, Andrea Tagliabue, Giulia Rossi, Davide Bochicchio**

Dipartimento di Fisica, Università di Genova, 16146 Genoa, Italy
* davide.bochicchio@unige.it

Amyloid plaques, aggregates mainly composed of amyloid-beta (Aβ) peptides surrounding neurons and cerebral blood vessels, are the hallmark of Alzheimer's disease, a widespread and still incurable neurological pathology.[1,2] The misfolding and overproduction of Aβ leads to its aggregation, although the mechanisms driving this phenomenon are currently subject to debate.[3,4] The prevailing hypothesis suggests that low molecular Aβ oligomers (AβOs) are particularly neurotoxic and pathogenic, surpassing mature Aβ fibrils in potency. Their primary target appears to be the neuronal cell membrane, with damage and disruption caused by AβOs considered a major contributor to neurotoxicity in Alzheimer's disease. Notably, AβOs' toxicity seems closely linked to their interaction with lipid rafts, [5—8] membrane regions where Aβ originates and eventually aggregates into AβOs, which in turn exert their deleterious effects through binding to these rafts.

Given the enormous complexity of the biological environment and the lack of spatial and temporal resolution of in-vivo experimental techniques, molecular models and simulations have become increasingly important for investigating the mechanisms underlying Aβ aggregation pathways. Herein, we report on our efforts in performing atomistic molecular dynamics simulations, complemented with enhanced sampling techniques, with the aim of (i) shedding light on the mechanisms underlying the Aβ-42 peptide aggregation in oligomers and small fibrills, and (ii) building and finely tuning an ad-hoc coarse-grained model of Aβ-42 with the aim to reproduce at best the peptide conformations and the aggregation-driven structural transitions observed at the atomistic level. Thanks to the CG model, we anticipate setting up simulations capable of spanning the necessary time (tens of μs) and length (tens of nm) scales needed to simulate the interaction between AβOs and complex model neuronal membranes containing lipid rafts.

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IN-SILICO EXPLORATION OF THE ALLOSTERIC MECHANISM IN MULTI-DOMAIN PROTEINS: THE CASE OF TYROSINE PHOSPHATASE SHP2

Paolo Calligari,[1] Mattia Carvetta,[1] Gianfranco Bocchinfuso, [1] and Lorenzo Stella[1]

[1]Department of Chemical Science and Technologies – Tor Vergata University of Rome, 00133 Rome, Italy
paolo.callligari@uniroma2.it

The SHP2 phosphatase plays a crucial role in mediating signal transduction downstream of various receptor tyrosine kinases. Mutations in the *PTPN11* gene, responsible for encoding SHP2, have been associated with cancer and developmental disorders.[1,2] The structure of SHP2 includes two Src homology 2 domains (N-SH2 and C-SH2), followed by a protein tyrosine phosphatase (PTP) domain, containing the catalytic site. In its inactive state, SHP2 adopts a closed autoinhibited conformation where the catalytic site of the PTP domain is obstructed by the N-SH2 domain. Upon interaction with its partners, SHP2 undergoes a significant conformational change, leading to its activation. SHP2 activation is mediated by the association between its SH2 domains and partners containing amino acid motifs comprising phosphotyrosine residues.

The activation mechanism of large systems like SHP2 poses computational challenges due to extensive domain motions and slow interconversion rates between inactive and active states (here on the order of 10^{-1} seconds). This study presents our findings from various enhanced sampling techniques (such as replica exchange molecular dynamics and metadynamics) and coarse-grained simulations using the Martini model.[3] We will discuss putative activation pathways and characterize the properties of the active SHP2 state, drawing insights from computational and experimental evidence.

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IN SILICO STUDY OF CHITOSAN INTERACTIONS WITH LIPID BILAYERS

Sonia Cambiaso[1], Roshan Shrestha[2], Hafez Razmazma[3], Davide Bochicchio[1], Giulia Rossi[1], Luca Monticelli[2]

[1] University of Genova, Dept of Physics, Genoa (IT), [2] Molecular Microbiology and Structural Biochemistry, CNRS, University of Lyon (FR), [3] University of Montpellier (FR)

This work has been developed in the framework of the H2020 SUNSHINE project (No 952924). Chitin, made of N-acetylglucosamine units, is one of the most abundant natural polysaccharides, synthesized by living organisms, such as arthropods, cell walls of fungi, and yeast. Its properties, including low nanotoxicity and biodegradability, make it ideal for biomedical and pharmaceutical applications, as well as for treating industrial pollutants. Chitosan is a cationic polymer derived from chitin partial deacetylation. It consists of a random distribution of β-(1→4) D-glucosamine (GlcN) and its acetylated derivative (GlcNAc). Polyelectrolyte complexes made of chitosan with proteins, polyanions, or DNA, have promising applications as advanced materials in transmucosal drug delivery [1] and other industrial applications. Molecular simulations are valuable tools for understanding how chain composition and solution physicochemical conditions influence polymer solubility, size, flexibility, and aggregation. To this purpose, we developed a coarse-grained model of chitin and chitosan compatible with the last release of the popular Martini forcefield [2], which allows the simulation of complex, multi-component systems with submolecular resolution while retaining a high degree of chemical specificity. Our chitosan model can accurately reproduce the structural features of polymer chains with different degrees of acetylation and charge states, which depend on the pH of the solution. The transferability of the model between different solvent environments allows the simulation of several applications involving chitin and chitosan. In the framework of the H2020 SUNSHINE project, we simulated a new multi-component nanomaterial (MCNM) consisting of graphene oxide flakes functionalized by chitosan and dispersed in a polyamide matrix. In the automotive sector, this innovative MCNM provides superior mechanical and flame-retardant properties, offering a non-toxic alternative to halogenated compounds. Our molecular simulations investigate its mechanical properties and the interactions with graphene oxide and with biological membranes, to gain insight into potential mechanisms of biological activity.

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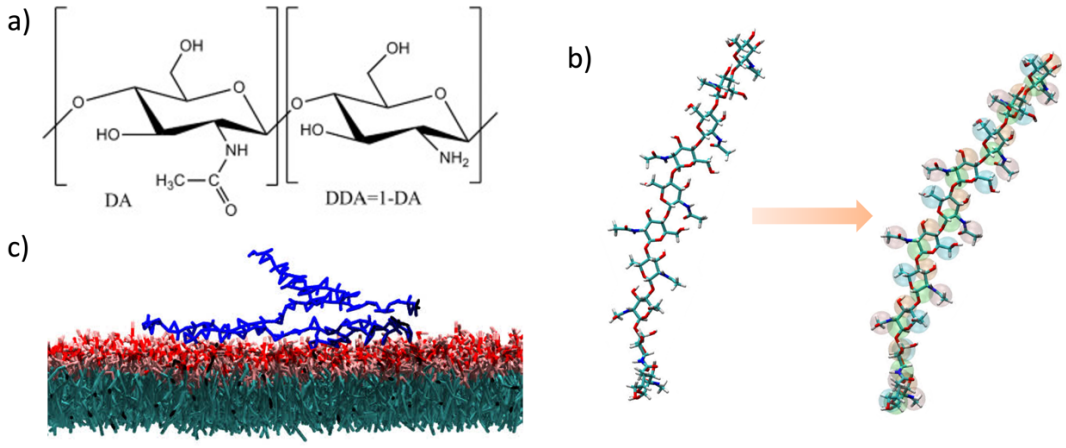

Figure 1: a) Chitosan, made of a random distribution of GlcN and GlcNAc monomers. b) atomistic (left) and coarse-grained model (right) of chitosan. c) interaction of chitosan (blue) with a lipid monolayer.

CHOLESTEROL-CONTAINING LIPOSOMES DECORATED WITH AU NANOPARTICLES AS MINIMAL TUNABLE FUSION MACHINERY

Ester Canepa[1,2] Davide Bochicchio[1], Giorgia Brosio,[1] Paulo Henrique Jacob Silva,[3] Francesco Stellacci[1], Silvia Dante[2], Giulia Rossi[1], Annalisa Relini[1]

1. Department of Physics, University of Genoa, Via Dodecaneso 33, Genoa 16146, Italy
2. Materials Characterization Facility, Istituto Italiano di Tecnologia, Via Morego 30, Genova 16163, Italy
3. Institute of Materials Science & Engineering, École Polytechnique Fédérale de Lausanne, Lausanne 1015, Switzerland
ester.canepa@edu.unige.it – ester.canepa@cbni.ucd.ie

Membrane fusion is essential for the basal functionality of eukaryotic cells. Synaptic transmission, membrane trafficking, and subcellular cargo transport are some physiological events involving rapid and selective membrane fusion. Defects in membrane fusion machinery may result in severe biological damage, including disorders in neuronal synapses. In physiological conditions, fusion events are regulated by a wide range of highly specialised proteins, operating with finely tuned local lipid composition and ionic environment. Fusogenic proteins, assisted by membrane cholesterol and calcium ions, provide the mechanical energy necessary to achieve vesicle fusion in neurotransmission. Similar cooperative effects must be explored when developing novel supramolecular systems mimicking controlled membrane fusion. Here we show that synthetic liposomes composed of cholesterol and 1,2-dioleoyl-sn-glycero-3-phosphocholine, and decorated with membrane-embedded amphiphilic Au nanoparticles, can act as a minimal tunable fusion machinery.1 Our Au nanoparticles have a monodisperse core diameter of 2.4 nm and are known to spontaneously and non-disruptively interact with the liposome lipid bilayer.2-4 Liposome fusion is triggered by divalent ions (Ca2+ or Mg2+), while the number of fusion events dramatically changes with, and can be finely tuned by, the liposome cholesterol content (0÷40 mol%). We combine dissipative quartz-crystal-microbalance (QCM-D), real-time fluorescence assays (vesicle content and FRET-based lipid mixing), and small-angle X-ray scattering (SAXS) with molecular dynamics (MD) at coarse-grained (CG) resolution, revealing new mechanistic details on the fusogenic activity of amphiphilic Au nanoparticles and demonstrating the ability of these synthetic nanomaterials to induce controlled vesicle fusion regardless of the divalent ion used. The results provide a novel contribution to developing new artificial fusogenic agents for next-generation biomedical applications that require tight control of the rate of fusion events (e.g., artificial synapses and targeted drug delivery).

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Biomimetic hybrid vesicles for cancer targeting

Giorgia Ailuno[1], Alice Balboni[1], Paolo Canepa[2], Andrea Lagomarsino[2], Elena Angeli[2], Claudio Canale[2], Ornella Cavalleri[2], Katia Cortese[3], Sara Baldassari[1], Federica Barbieri[4], Tullio Florio[4], Gabriele Caviglioli[1]

1 Department of Pharmacy - University of Genoa, Italy
2 Department of Physics - University of Genoa, Italy
3 Department of Experimental Medicine, Human Anatomy - University of Genoa, Italy
4 Department of Internal Medicine - University of Genoa, Italy paolocanepa@unige.it

Glioblastoma (GBM), an aggressive primary tumor of the central nervous system, currently lacks curative treatment options, as chemotherapy effectiveness is hampered by the inadequate accumulation of drugs within the tumor[1-2].

Exosomes, small vesicles released by various cell types, have recently garnered significant attention due to their capacity to transport biomolecules, their targeting ability, and their low potential for triggering immune responses[3]. However, their large-scale production for clinical applications is still triggering due to the limited yield of naturally isolated and the difficult in obtaining high levels purification[4]. Consequently, the development of bioinspired exosome-mimetics with enhanced yield and consistency holds promise for drug delivery purposes.

In this work, we prepared cell-derived nanovesicles (CMVs) and hybrid biomimetic liposomes using purified cell membrane suspensions from glioblastoma cell. The physical-chemical characteristics of CMVs were examined, and their preferential uptake by parent cells compared to healthy oligodendrocytes was assessed using confocal microscopy. The hybridization with artificial liposomes was confirmed through Förster Resonance Energy Transfer (FRET) spectroscopy.

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ANTI-AMYLOID AND ANTI-OXIDATIVE PROPERTIES OF MORINGA OLEIFERA EXTRACTS AS STRATEGIC PHYTO-THERAPY FOR ALZHEIMER DISEASE

Rita Carrotta,[1] Silvia Vilasi,[1] Maria Assunta Costa,[1] Rosa Passantino,[1] Fabio Librizzi,[1] Vincenzo Martorana,[1] Carla Buzzanca,[2] Vita di Stefano,[2] Maria Grazia Ortore,[3] Silvia Piccirillo[4], Alessandra Preziuso[4], Simona Magi,[4] and Maria Rosalia Mangione[1]

1 Institute of Biophysics, National Research Council, Palermo, Italy;
2 Department of Science and Biological, Chemical and Pharmaceutical Technologies STEBICEF, University of Palermo, Italy;
3 Department of Life and Environmental Sciences, Marche Polytechnic University, Ancona, Italy;
4 D Department of Biomedical Sciences and Public Health, School of Medicine, Marche Polytechnic University, Ancona, Italy;

Alzheimer's Disease (AD), the most common form of dementia, is a neurodegenerative pathology characterized by the accumulation of extracellular amyloid plaques composed by well-ordered and β-sheet rich fibers of the Amyloid β peptide (Aβ). Although Aβ fibers are the hallmark of AD, consistent evidences rather indicate that oligomers of Aβ act as toxins, responsible for oxidative stress and mitochondrial dysfunction, causing neurodegenerative damage. Many efforts have been focused therefore on the individuation of molecules capable to inhibit the pathological aggregation and/or reduce ROS production. An increasing number of studies, *in vitro* and *in vivo*, highlight that natural polyphenols can work both as amyloid inhibitors and antioxidants, thus making them very attractive for the development of effective phyto-therapeutic prevention strategies. A plant attracting much interest in the last years for its therapeutic potentiality and richness on polyphenols is the Moringa Oleifera, a fast-growing tree originating from the sub-Himalayan area.

In this work Moringa Oleifera aqueous extracts (MOAE) have been characterized for their polyphenolic content, anti-oxidative and free radical scavenging capacity. MOAE inhibitor effect was studied on the in vitro aggregation kinetics of $A\beta_{1-42}$ peptide. Results show a strong anti-amyloid effect at very low concentration of MOAE, as seen by ThT assay, circular dichroism and atomic force microscopy. Interestingly, in the range explored, the lag time of fibrillation was inversely correlated with polyphenolic concentration unrevealing an intriguing natural aggregation process of polyphenols. These results suggest that after an initial shielding of the peptides, later on polyphenols/peptides aggregates can nucleate an amyloid-like aggregation, while overall maintaining a reduction of fibrillation. Finally, retinoic acid-differentiated SH-SY5Y cells, challenged with glyceraldehyde (GA), were used as an AD cell model. The MOAE antioxidant activity and their efficacy to inhibit the production of $A\beta_{1-42}$, stimulated by GA, were tested. Treated cells showed an increase on cell viability in comparison with the controls. Moreover, mitochondrial ROS and GA-stimulated $A\beta_{1-42}$ production were reduced in cells exposed to MOAE.

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TARGETING BIR-MEDIATED ONCO-PPIS: RATIONAL DESIGN OF NF-*K*B MODULATORS

Paolo Cocomazzi, Michele Fiore, Enrico Millo, Elisa Fagnani, Francesco Bonì, Federica Cossu

1 AIBF-CNR, Istituto di Biofisica sede di Milano, via A. Corti, 12, 20133 Milano Italy
2 BIBF-CNR, Istituto di Biofisica sede di Genova, via De Marini,6 Torre di Francia, 16149 Genova Italy
3 Department of Experimental Medicine, Section of Biochemistry, University of Genoa, Viale Benedetto XV 1, 16132 Genova, Italy
Paolo.cocomazzi@ibf.cnr.it

Over-expression of inhibitors of apoptosis proteins (IAPs) family enhances cell survival and resistance to chemotherapics. IAPs-mediated complexes ubiquitylate substrates regulating NF-κB pathway. Type I BIR (Baculovirus IAP repeat) domains of IAPs are pivotal for the assembly of such complexes. IAPs' Type-II BIRs interact with caspases to inhibit cell-death, or with SmacDIABLO for apoptosis restoration. Type II BIRs-directed therapies, Smac-mimetics (SMs), relieve caspases from inhibition by X-linked IAP and induce cIAPs (cellular IAP1 and 2) auto-ubiquitination and degradation. Despite IAPs-directed therapies target pockets or hotspots on isolated, globularly structured BIR domains, their relative positioning within the entire IAP molecule is the key for various pro-survival roles. Since SMs treatment produces divergent effects, such as cIAP2 upregulation leading to cell survival, full length IAPs structural information is necessary.

Our aim is to target BIR-mediated onco PPIs to (i) develop/improve IAPs-targeting therapies and (ii) unravel the molecular determinants of the action of IAPs or IAPs-inhibitors. We have already identified compounds binding BIR1-mediated PPI surfaces and we characterized a library of more than 50 novel putative anti-cancer molecules *in vitro*, analysing the ability to bind target proteins and to induce cell death in a panel of four tumor cell lines (prostate cancer, triple negative adenocarcinoma, non-small cell lung cancer). We selected 2-3 candidates with best profiles to elucidate pro-death mechanisms. We are currently producing the recombinant form of different FL-IAP homologues and protein partners as TRAF2 and TAB1, for biophysical and biochemical investigations. The modulation of pro-survival complexes regulating the NF-κB pathway, as the ones mediated by IAPs, can be the strategy to overcome cases of resistance to current IAPs-targeting chemotherapics.

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COMMERCIAL SERS SUBSTRATES FOR THE DETECTION OF BIOLOGICAL MOLECULES

Pauline Conigliaro,[1,2] Stefano Prato,[3] Barbara Troian,[3] Anton Naumenko[3] and Ines Delfino[1]

[1] Dipartimento di Scienze Ecologiche e Biologiche, Università degli Studi della Tuscia, I-01100 Viterbo, Italy;
[2] Dipartimento di Economia, Ingegneria, Società e Impresa, Università degli Studi della Tuscia, I-01100 Viterbo, Italy;
[3] A.P.E. Research S.r.l., Area Science Park, Basovizza, 34149 Trieste, Italy;

Surface-enhanced Raman spectroscopy (SERS) is a non-destructive and sensitive technique, which has raised the attention of the scientific community for numerous applications, such as the detection and identification of biological materials and molecules of environmental interest.[1] The basis of this technique is an extension of normal Raman spectroscopy, given by the electronic and chemical interactions between the excitation laser, the analyte of interest, and the metallic substrate (e.g. silver/copper/gold surfaces) used.[2] The properties of these substrates are crucial to ensure the reliability and sensitivity of the experiments, as well as, the reproducibility of the measurements and the simultaneous detection of classes of molecules with different chemical characteristics (polarity, molecular weight, etc).[3]
To date, one of the simplest strategies for fabricating SERS substrates is the use of colloidal nanoparticle solutions[4] or the purchase of commercial substrates. In this work, we present measurements to examine the characteristics of two commercial SERS substrates, each featuring nanostructures and designed to perform optimally at an excitation wavelength of 785 nm. Aspirin C was used as a representative molecule to evaluate the suitability of these substrates for SERS analysis of biological molecules, due to its distinctive spectral characteristics. It comes in effervescent tablets and includes acetylsalicylic acid and ascorbic acid as active constituents, renowned mainly for their analgesic and anti-inflammatory properties.[5] Overall, this investigation helps advance our understanding of SERS substrates and their potential applications in bioanalytical and environmental chemistry.

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NEW INSIGHTS INTO THE ROLE OF CLC-6 AND CLC-7 IN THE LATE ENDOSOMES AND LYSOSOMES: A FUNCTIONAL IN VITRO STUDY

Maria Antonietta Coppola[1], Abraham Tettey-Matey[1], Paola Imbrici[2] , Antonella Liantonio[2] , Paola Gavazzo[1] , Peying Fong[3] , Michael Pusch[1]

Institute of Biophysics, CNR, Genoa, Italy
Department of Pharmacy–Drug Sciences, University of Bari "Aldo Moro", Bari, Italy
Department of Anatomy and Physiology, Kansas State University College of Veterinary Medicine, Manhattan, KS, USA
mary88.cop@gmail.com

Belonging to a distinct branch of the CLC family, ClC-6 and ClC-7 are vesicular $2Cl^-/H^+$ antiporters localized in late endosomes and lysosomes respectively, where they are important for ionic homeostasis of these organelles (1). Both are predominantly expressed in nervous tissue, whereas ClC-7 is also found in the ruffled border of osteoclasts. However, their physiological roles need to be clarified.

Here, to gain new insight into the role of ClC-6 and ClC-7 in their compartments, we measured Cl^-- and pH-dependences of ClC-6, ClC-7 as well as mutants causing human diseases. Among these, ClC- 6^{Y553C} (2,3) and ClC-7^{Y715C} (4) were compared to their corresponding ClC-7^{Y577C} and ClC-6^{Y781C} constructs over-expressed in HEK293 cells and using whole cell patch clamp. It has to be kept in mind that the extracellular solution corresponds to the intraluminal endo-lysosomal compartment.

Lowering extracellular Cl^- reduced ClC-6 transport activity, whereas it increased activity of ClC-7. In ClC-7, the activation mediated by low Cl^- persisted under acidic pH_{ext}. Interestingly, ClC-7^{Y577C} recapitulated the GoF effect seen in the corresponding ClC-6^{Y553C}. Additionally, ClC-6^{Y781C} showed similar faster kinetics to those observed in ClC-7^{Y715C}. Interestingly, lowering luminal chloride concentration had less effect on the activation of the ClC-7^{R760Q} variant (5). Of note, all mutants overall reflected WT behaviors.

These findings show that ClC-6 and ClC-7 have non-overlapping physiological roles in their respective compartments. We further propose that the inhibition of ClC-7 by luminal Cl^- serves to limit excessive intraluminal Cl^- accumulation, thereby preventing osmotic overload of lysosomes.

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IMAGING CHROMATIN COMPACTION IN CELLS OF WHOLE PLANARIANS: A STEP TOWARDS THE CHARACTERIZATION OF EPIGENETIC MEMORY IN LOWER EUKARYOTES

Sara Corti,[1] Patrizia Guidi,[2] Leonardo Rossi,[2] Ranieri Bizzarri[1,2]

[1]Department. of Surgical, Medical and Molecular Pathology and Critical Care Medicine, University of Pisa, Pisa, Italy.
[2]Department of Clinical and Experimental Medicine, University of Pisa, Pisa, Italy
saracorti@live.com, ranieri.bizzrari@unipi.it

From the emergence of what we call life, chemical cognition of the environment has played a crucial role in the ability of any organism to adapt to its environment and increase its reproductive fitness. Yet, the discovery of *learning*, i.e. a persistent and adaptive modification of an organism's behavior as a function of its experience, has been a major breakthrough in the history of evolution. Indeed, learning (and therefore, memory) allows organism to adapt to their environment on time scales much faster than genetic selection driven by the sole chemical cognition. Learning is usually attributed to multicellular organism, through the existence of a nervous system and its inherent synaptic plasticity. Nonetheless, in the last fifty years some researchers questioned the existence of learning capabilities also in unicellular organisms such as protists, or in multicellular organisms such as planarians through processes largely independent of the presence of a nervous system. Thus, the question looms: can single cells carry out information processing traditionally attributed to networks of cells? Albeit the answer is still unknown, recent progresses in functional imaging have shown that the biochemical foundation and regulation of cell's processes are intimately linked to the nanoscale arrangement of biomolecules such as transcription factors, motor proteins, and RNA. For this aim, we started out to explore the chromatin compaction in the cells of planarians by taking advantage of a FRET-based imaging system which leverages cell-permeant dyes Hoechst and Syto13 [1]. Albeit these constitute preliminary results, we found out an interesting eterogeneity in the chromatin compaction of planarian cells which could be attributed to the different stages of trascriptional program of the cell themselves. Additionally, our method demonstrate for the first time that the chromatin of single cells of the whole (fixed) animal can be effectively imaged, thus enabling further studies where the chromatin compaction can be correlated with the expression of selected genes.

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EFFECTS OF THE MEMBRANE'S COMPOSITION ON THE BERBERINE PERMEABILITY IN LIPOSOMES

Flavio Costa,[1] Giorgia Giorgini,[2] Cristina Minnelli,[2] Giovanna Mobbili,[2] Carlo Guardiani,[1] Alberto Giacomello,[1] and Roberta Galeazzi [2]

[1]Dipartimento di Ingegneria Meccanica e Aerospaziale, Sapienza Università di Roma, Via Eudossiana 18, 00184, Rome, Italy
[2]Department of Life and Environmental Sciences, Marche Polytechnic University, Via Brecce Bianche, 60131, Ancona, Italy
f.costa@uniroma1.it

Berberine (BBR) is a natural molecule with several pharmacological properties that represents a novel strategy to prevent the antibiotic resistance in gram-negative bacteria (1-2). However, its oral bioavailability is poor, thus resulting into an impaired absorption and efficacy in humans (3). In the past, liposomes have been shown to enhance the drug's availability, representing a smart delivery system to target tissues without negative side effects (4). In this work, a combination of experimental and computational assays was used to describe how the membrane composition of liposomes influence their capability to encapsulate BBR. Firstly, the encapsulation efficiency parameter was described in presence of several membrane compositions, revealing that the cholesteryl hemisuccinate and, more slightly, cholesterol enhance it for BBR. Then, the physical basis of such parameter has been clarified, lying on the capability of membranes to attract, i.e., to adsorb, the molecules onto their surface. Overall, these findings suggest a rational strategy to maximize the encapsulation efficiency of liposomes by using negatively charged lipids, thus representing the basis for designing delivery systems for BBR useful to treat, mostly, the antibiotic resistance.

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THE SUBTLE ROLE OF POLYHOMEOTIC POLYCOMB PROTEINS IN DETERMINING BIOMOLECULAR CONDENSATES IN THE CELL NUCLEUS

Sofia Cristiani,[1] Alberto Leggio,[1] Benedetta Masciulli,[1] Tiziano Salvadori,[1] Vittoria Carnicelli,[1] Barbara Storti,[2] Ranieri Bizzarri[1,2]

1 Department. of Surgical, Medical and Molecular Pathology and Critical Care Medicine, University of Pisa, Pisa, Italy.
2 NEST Scuola Normale Superiore & Istituto Nanoscienze-CNR, Pisa, Italy
s.cristiani2@studenti.unipi.it, a.leggio@studenti.unipi.it, benedetta.masciulli98@gmail.com,

Polycomb Group (PcG) proteins has emerged as critical factors in epigenetic inheritance, maintaining cell identity by repressing genes involved in development through mechanisms such as H3K27me3 marking and chromatin compaction [1]. Indeed, the presence of PcG protein homologues across a wide range of species, from plants to humans, indicates their evolutionary conservation, highlighting their fundamental role in biology. The discovery that PcG proteins aggregate into cellular structures known as PcG bodies, combined with their rich content of intrinsically disordered regions (IDRs) and protein interaction motifs, supports the notion that their assembly could be facilitated through liquid-liquid phase separation (LLPS), i.e. that PcG bodies are in most cases biomolecular condensates and this physical state may play a crucial role in sustaining gene repression [2, 3]. Among PcG proteins, a special role is played by polyhomeotic-like proteins (PHC1, PHC2, PHC3), which participate to the transcriptional-repressing activity of Polycomb Repressor Complex 1 (PRC1). Due to the presence of a Sterile Alpha Motif (SAM) at C-terminus, and Intrinsically Disordered Regions at N-terminus, it is believed that PHCs may create crosslinks that confine chromatin into meso/nanoscale condensate domains [3]. Despite the overexpression/downregulation of PHCs in several tumors, which highlights how PHCs are crucial factors in determining the cell's phenotype, the actual role of these proteins in the remodeling of chromatin is obscure. Our group set out to fully characterize the role of PHCs in physiological and tumoral cells by combining traditional biochemistry and molecular biology methods with high-resolution/nanoscopy microscopy. In particular, we studied the morphological and nuclear distribution features of PcG bodies containing PHC2 and PHC3 in *glioblastoma multiforme* Non-Small Cell Lung Cancer, and thyrocyte (physiological and tumoral) cell lines, highlighting their condensate properties. By means of super-resolution techniques we were also able to generate an interaction map between PHC2-3 and other polycomb components, and to link them to physiological and non-physiological states. Finally, drug targeting methods, such as RNA interference approaches, were envisaged to silence these genes in tumoral cell and restore more physiological phenotypes.

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SUPER-RESOLUTION STORM MICROSCOPY AS A NEW TOOL TO STUDY EXTRACELLULAR VESCICLES.

Martina De Felice[1], Alberto Morgantini[1*], Rebecca Mordini[1*], L. Germelli[2], L.Ceccarelli[2], M.Costa[5,6], A. Cattaneo [6,7], C. Martini[2,3], L. Marchetti[2,3], F. Cella Zanacchi[1,3,4]*

[1]Department of Physics, University of Pisa, Pisa, Italy27 Pisa, Italy
[2]Department of Pharmacy, University of Pisa, Pisa, Italy
[3]Center for Instrumental Sharing of the University of Pisa, Pisa, (CISUP)
[4]Nanoscopy and NIC@IIT, Istituto Italiano di Tecnologia, 16152 Genoa, Italy
[5]Institute of Neuroscience, Italian National Research Council (IN-CNR), 56124 Pisa, Italy
[6] Bio@SNS Laboratory, Scuola Normale Superiore, Pisa 56124, Italy.
[7]European Brain Research Institute "Rita Levi-Montalcini" (EBRI), 00161 Rome, Italy
*The authors contribute equally.

Extracellular vesicles (EVs), consisting of bi-layer lipid vesicles, are released from cells to extracellular space and emerged as fundamental mediators between cells in health and disease[1,2].
Given their physiological function as carriers for RNA, DNA, proteins, and small molecules, in recent years EVs have been of interest as new promising tools for therapeutic RNA delivery.[3]
In this context, the study of EVs delivery and their interactions with cellular biomolecules is fundamental; however, their small size (150-200 nm) and lack of improved imaging techniques makes their localization and interactions studies challenging.
For this reason, Stochastic Optical Reconstruction Microscopy (STORM) is used in this study to overcome the diffraction limit of conventional light microscopy, providing a quantitative tool[4] to localize ad quantify the abundance of two neurotrophic receptors, namely TrkA and p75NTR receptors, in EVs. We compared four different EVs types, produced by CRISPR-Cas9 edited PC12 cell lines: knockout for TrkA, p75NTR or both receptors compared to wild-type PC12.
The use of STORM imaging allowed us to quantify the number of localized proteins in EVs, the dimension of the EVs and the "density" as the ratio between the molecules number and the clusters area, either for TrkA or p75NTR in the four different type of EVs.
STORM microscopy in this study is proposed as an helpful imaging technique in quantify TrkA and p75NTR carried by EVs and it may support in the future the rational design of a targeted delivery strategy to be exploited in pathological/degenerative conditions in which TrkA and p75NTR are involved. Furthermore, quantitative super-resolution may represent in the future a helpful tool to characterize, at the nanoscale level, EVs as nano-templates for targeted delivery of DNA/ RNA formulations.
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APTAMER-PROTEIN BINDING QUANTIFIED BY FLUORESCENCE CORRELATION SPECTROSCOPY

David Porciani,[1] Manuela Maria Alampi,[2] Stefania Abbruzzetti,[2] Cristiano Viappiani,[2] and Pietro Delcanale[2]

1MU Bond Life Sciences Center, University of Missouri-Columbia, 1201 Rollins Street, Columbia, Missouri 65211-7310, USA (present address: SomaLogic, Inc., La Jolla, CA 92037, USA)

2Dipartimento di Scienze Matematiche, Fisiche e Informatiche, Università di Parma, Parco Area delle Scienze 7A, Parma 43124, Italy;

pietro.delcanale@unipr.it

Aptamers are folded oligonucleotides that are artificially selected to specifically recognize a target molecule and are therefore emerging as a potential alternative to antibodies for many applications.

A quantitative measurement of aptamer-target binding is decisive for the rational selection and optimization of functional aptamer molecules. In addition to the measurement of equilibrium affinity for the target, association and dissociation kinetics and the interaction with non-target components, such as serum proteins, are often critical for aptamer functionality. Still, a quantitative evaluation of these parameters with biophysical techniques is not trivial and often poses limiting requirements, for example, the need for surface immobilization of molecules, the use of large amounts of products, or a poor compatibility with biologically relevant conditions.

In this work, an experimental method based on Fluorescence Correlation Spectroscopy (FCS) was used to characterize *in vitro* the binding of three different DNA aptamers (CLN3, AS1411 and 36t), labelled with a fluorophore, with their respective soluble recombinant target proteins (c-Met, nucleolin and PDGF-BB), that significantly differ in size, binding strength, and biological function.[1] Using FCS, we quantified both equilibrium affinity and binding kinetics within simple homogeneous solutions. Additionally, the behaviour of the CLN3 aptamer exposed to physiological concentrations (micro to milli-molar) of the most abundant serum proteins, namely albumin, immunoglobulin, fibrinogen, and transferrin, was investigated with FCS. The results show that the aptamer has negligible interactions with these species and that highly concentrated serum proteins do not affect the observed affinity for the target. These findings introduce the use of FCS in the context of a rational development of functional protein-targeting aptamers.

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LIPOSOMES, PROTEIN DELIVERY AND CONTROLLED MODULATION OF THE PHOTOTRANSDUCTION CASCADE

Sabrina Asteriti,[1,2] Valerio Marino,[1] Anna Avesani,[1] Amedeo Biasi,[1] Giuditta Dal Cortivo,[1] Lorenzo Cangiano,[2] and Daniele Dell'Orco[1]

[1]Department of Neurosciences, Biomedicine and Movement Sciences, Section of Biological Chemistry, University of Verona, 37134 Verona, Italy
[2] Department of Translational Research, University of Pisa, 56123 Pisa, Italy
daniele.dellorco@univr.it

Vision in vertebrates begins with light absorption by the G protein-coupled receptor (rhod)opsin, which triggers the phototransduction cascade in photoreceptors. The viability of both rod and cone photoreceptors depends closely on homeostatic levels of the second messengers cGMP and Ca^{2+}, disruption of which leads to severe retinal dystrophies, often associated with mutations in the genes involved in the phototrasduction cascade. Based on our previous results on protein delivery to the mouse retina via lipid nanovesicles,[1] we explored the possibility of modulating the phototransduction cascade in mouse rods using direct or liposome-mediated delivery of a recombinant protein crucial for regulating the interplay of second messengers in the outer segments of photoreceptors.[2] The effects of administration of free and liposome-encapsulated human guanylate cyclase activating protein (GCAP1) were compared in biological systems of increasing complexity (*in cyto*, *ex vivo*, and *in vivo*). Analysis of protein biodistribution using fluorescently labeled GCAP1 (far-red emission) and direct measurement of the functional alteration of rod photoresponses show that exogenous GCAP1 protein is fully incorporated into the mouse retina and photoreceptor outer segments. Furthermore, delivery of a GCAP1 variant carrying a point mutation associated with cone-rod dystrophy in human p.(E111V), which prevents the physiological conformational change required to regulate the target enzyme,[2] results in a disease-like electrophysiological phenotype as assessed by a novel *ex-vivo* electroretinography apparatus,[3] consistent with constitutive activation of the retinal guanylate cyclase. Our study shows that direct protein delivery and liposome-mediated delivery are powerful tools for targeting signaling cascades in neuronal cells, which could be particularly important for the treatment of autosomal dominant genetic diseases.

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THE EUPRAXIA PHOTON BEAMS: ULTRA-BRIGHT LIGHT PULSES FOR IMAGING AND SPECTROSCOPY

Emiliano De Santis,[1] on behalf of the EuPRAXIA collaboration

[1]University of Rome Tor Vergata & INFN, Rome, 00133, Italy
emiliano.desantis@physics.uu.se

EuPRAXIA is a leading European project aimed at the development of a dedicated, groundbreaking, ultra-compact accelerator research infrastructure based on novel plasma acceleration concepts. EuPRAXIA is an important intermediate step between proof-of-principle experiments and ground-breaking, ultra-compact accelerators for photon science, structural biology, particle physics detector development, materials science, medical imaging, radiation therapy, and industrial applications.
The INFN Laboratori Nazionali di Frascati will host one of the EuPRAXIA research infrastructures and will be equipped with an X-band LINAC followed by a plasma wakefield acceleration stage driving the first plasma-based free electron laser (FEL).[1] The FEL will be characterized by a small footprint and will deliver ultra-bright photon pulses for experiments in the water window (3-5 nm) to the users community.[2] The facility will host a second photon beamline with seeded FEL pulses in the range between 50 and 180 nm.[3]
In addition, EuAPS, the EuPRAXIA Advanced Photon Source, will exploit the ultra-short X-ray pulses emitted by the electrons accelerated in plasmas wakefields, the so-called betatron radiation, in a dedicated beamline.[4,5,6]
Here we describe the foreseen applications of the FEL and betatron photon sources, which include imaging and spectroscopy measurements on a variety of samples, both biological and inorganic, providing information about their structure and dynamical behavior.

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COMPUTATIONAL EVIDENCES OF A MISFOLDING EVENT IN AN AGGREGATION-PRONE LIGHT CHAIN IN AL AMYLOIDOSIS

Fausta Desantis,[1,2] Mattia Miotto,[2] Edoardo Milanetti,[3,2] Giancarlo Ruocco,[2,3] and Lorenzo Di Rienzo[2]

[1]The Open University Affiliated Research Center at Istituto Italiano di Tecnologia, Via Morego, 30, Genova, 1613, Italy
[2]Istituto Italiano di Tecnologia (IIT), Center for Life Nano & Neuro Science, Viale Regina Elena 291, Rome, 00161, Italy
[3]Sapienza University of Rome, Department of Physics, Piazzale Aldo Moro, 5, Rome, 00185, Italy
fausta.desantis@iit.it

Antibody light chain (AL) amyloidosis is a condition associated with the aggregation and deposition of immunoglobulin light chains in diverse tissues resulting in organ impairment[1]. The high susceptibility of antibodies to mutations makes AL amyloidosis strongly patient-specific. In this context, understanding the processes underlying fibrillization is crucial in the perspective of achieving prompt diagnoses and designing personalized therapeutics. Here, we focus on a particular patient-derived light chain, previously experimentally characterized[2,3]. We investigated the early phases of the aggregation pathway first through extensive full-atom molecular dynamics simulations on the germ-line non-aggregating monomer and on its mutant aggregation-prone counterpart, then considering the dimerization process through docking and further molecular dynamics simulations, proposing a pathological dimeric structure. The simulations on the monomers revealed significant changes in the mutant light chain structure with the exposure of two hydrophobic residues (which remain buried throughout the entire germ-line's dynamics, instead). Based on this evidence we speculated that these could be part of a binding region leading to pathogenic dimerization. Molecular dynamics simulations on the docked structures allowed to find the most stable putative pathogenic dimer[4]. The results obtained could pave the way to the computational study of ensuing phases of light chains aggregation (e.g. involving coarse-grained simulations to observe oligomerization). Furthermore, extending the methods employed to other cases of aggregating light chains could uncover some common mechanisms driving misfolding and initial pathological association in AL amyloidosis. This could lead to the development of patient-specific drugs able to hinder protein assembly in the earliest stages.

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COMPUTATIONAL MODULATION OF COMPATIBILITY BETWEEN BIOMOLECULAR INTERFACES: THE IMPORTANCE IN PROTEIN DESIGN

Lorenzo Di Rienzo,[1] Mattia Miotto,[1] Edoardo Milanetti,[2,1] Giancarlo Ruocco,[1,2]

1 Center for Life Nano-& Neuro-Science, Istituto Italiano di Tecnologia, Viale Regina Elena 291, 00161 Rome, Italy.
2 Department of Physics, Sapienza University of Rome, Piazzale Aldo Moro 5, 00185 Rome, Italy. lorenzo.dirienzo@iit.it

The intricate network of interactions among biomolecules plays a pivotal role in regulating nearly all cellular functions. Molecular recognition between partners hinges on the precise adjustment of the physicochemical properties of the amino acids located on both sides of the interface. Indeed, even a single residue mutation can disrupt a molecular association, often resulting in a pathological alteration of a biochemical pathway [1]. Consequently, assessing the effects of amino acid substitutions on binding and custom designing molecular interfaces represent significant challenges in computational structural biophysics. By employing the orthonormal basis of Zernike polynomials to model the shape of the interaction surface, we delineate the geometry of binding sites using a compact ordered set of rotationally invariant descriptors. This facilitates a swift and efficient assessment of compatibility between interacting surfaces [2]. Moreover, by incorporating a concise model of chemical complementarity into the framework [3], we introduce an innovative approach for computational mutagenesis and optimization of protein interfaces. Utilizing Monte Carlo simulated annealing simulations where in each step an amino acid is substituted and the new properties evaluated, we can generate protein mutants with controlled molecular compatibilities. This method has broad applications, ranging from designing protein pockets capable of recognizing user-defined molecules to developing peptides that disrupt protein-protein interactions [4,5]. It demonstrates remarkable effectiveness in identifying a small set of amino acid substitutions capable of modulating the stability of corresponding molecular complexes.

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THE MINFLUX REVOLUTION OF MINIMAL PHOTON FLUXES AND POLARIZED LIGHT LABEL-FREE APPROACHES TO STUDY COMPLEX BIOMOLECULAR ORGANIZATIONS IN CELLS AT THE NANOSCALE.

Alberto Diaspro,[1,2] Elena Angeli[1], Paolo Bianchini,[2] Virginia Bazzurro,[1] Ranieri Bizzarri,[2,3,4] Simone Civita,[2,5] Francesca Cella Zanacchi,[6] Elena Gatta,[1] Matteo Mariangeli,[2,5] Marco Salerno.[1]

1 SEELIFE, Department of Physics, University of Genoa, Genoa, Italy;
2 Nanoscopy & NIC@IIT, CHT Erzelli, Istituto Italiano Tecnologia, Genoa, Italy;
3 Dept. of Surgical, Medical and Molecular Pathology and Critical Care Medicine, University of Pisa, Pisa, Italy;
4 NEST, Scuola Normale Superiore and Istituto Nanoscienze-CNR Pisa, Italy;
5 Department of Mathematical, Physical and Computer Sciences, University of Parma, Parma, Italy 6Physics Department "E. Fermi", University of Pisa, Pisa, Italy
diaspro@fisica.unige.it

Super resolved fluorescence optical microscopy enables the mapping of fluorophores that have been linked to molecules of interest using on/off state transitions as a key element of all diffraction-unlimited methods like STED, PALM/STORM[1], and the more recently introduced MINFLUX[2]. The challenges in nanoscale biophysics lie in devising experimental strategies optimized for deciphering biologically relevant mechanisms and obtaining quantitative information at the molecular scale. Exploiting minimal photon fluxes is based on a brand-new strategy in using a scanning doughnut-shaped beam that allows optical microscopy to enter the Angstrom spatial localization range[3]. This is the tip of an iceberg incorporating pioneering studies based on minimal light exposure[4] and a recent approach based on diffraction minima to resolve point scatterers at distances of 1/80 of the wavelength[5]. In parallel, the growth of label-free approaches[6] opens a new scenario for correlative multimodal optical microscopy at the nanoscale. Here, we present some examples of acquisition protocols and applications in biophysics at the nanoscale, focusing on MINFLUX, polarized label-free microscopy[7] and the cell nucleus. Moreover, we critically discuss the possible integration with artificial intelligence algorithms[8]. Partially funded by the European Union—Next Generation EU PNRR MUR Infrastructure - SEELIFE **(**B53C22001810006, IR0000023)

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IN VITRO CHARACTERIZATION OF A SCN2A VARIANT CAUSING EARLY INFANTILE ONSET ENCEPHALOPATHY USING HETEROLOGOUS CELL EXPRESSION AND NEURONS FROM IPS CELLS

Loretta Ferrera[1], Alessandra Ludovico[2], Antonella Riva[3], Lorenzo Muzzi[4], Giulia Lombardo[5], Lisastella Morinelli[6,7], Pasquale Striano[2,3], Anna Corradi[6,7], Federico Zara[1,2]

[1]Unit of Medical Genetics, IRCCS Istituto Giannina Gaslini, Genoa,Italy
[2]Department of Neurosciences, Rehabilitation, Ophthalmology, Genetics, Maternal and Child Health, University of Genoa, Genoa,Italy
[3]Pediatric Neurology and Muscular Disease Unit, IRCCS Istituto Giannina Gaslini, Genoa,Italy
[4]Department of Informatics, Bioengineering, Robotics and Systems Engineering, University of Genoa, Genoa, Italy
[5]General Pediatrics and Pediatric Acute and Emergency Unit, Neonatal Intensive Care Unit and Neonatal Accompaniment Unit, Azienda Ospedaliero-Universitaria Policlinico-San Marco, San Marco Hospital, University of Catania, Catania,Italy
[6]Department of Experimental Medicine, University of Genoa, Genoa,Italy
[7]Department of Neuroscience and Brain Technologies, Fondazione Istituto Italiano di Tecnologia, Genoa,Italy
lorettaferrera@gaslini.org

The SCN2A gene, coding for the Nav1.2 sodium channel, is crucial in neuronal action potential dynamics and is implicated in various epileptic disorders[1,2]. We examine the A1659V SCN2A variant, discovered in a patient with severe early-infantile onset encephalopathy, unresponsive to sodium channel-blocking treatments. This study aims to elucidate the variant's functional impact using in vitro models. The sequencing from lymphocytes and then confirmed on fibroblasts revealed 15% of mosaicism. We inserted A1659V mutation in a stabilized SCN2A plasmid by site-directed mutagenesis and showed no effect on channel expression. Biophysical properties of A1659V variant have been characterized in vitro using heterologous expression in Hek293 cells with patch clamp technique. In channels carrying the mutation we discovered a reduction of the peak current in addition to a shift of half activation voltage towards more negative values. We confirmed the hypothesis that the variant is a loss of function instead of a gain of function, as it generally happens for patients with early onset seizures, such this one.
We cultured patient's fibroblast in order to reprogram them in iPSC. After the transduction with Sendai Virus, we obtained around 50 clones which have been characterized to discern the WT from the heterozygous ones, due to the mosaicism starting point. We selected the best clones, both WT and with A1659V variant, and completed the characterization with: RT-PCR to check the removal of viral genes, immunofluorescence to confirm the presence of the staminal factors and Array CGH to identify potential abnormalities. From this point, we will differentiate iPSC into glutamatergic neurons through neurogenin protocol to test them on MicroElectrode Assay (MEA). This study provides valuable insights into the SCN2A A1659V variant's functional consequences, highlighting the utility of heterologous cell and iPSC-derived neuronal models in understanding complex neurological disorders.

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PRELIMINARY DATA ON HUMAN SLC45A1 CHARACTERIZATION

Margherita Festa[1], Abraham Tettey-Matey[1], Alice Giusto[1], Michael Pusch[1]

[1]IBF, Istituto di Biofisica - CNR, via De Marini 6, 16149 Genova, Italy
margherita.festa@ibf.cnr.it

The putative neuronal glucose transporter SLC45A1 (solute carrier family 45 member 1/ DNB5 deleted in neuroblastoma 5/ PAST-A proton associated sugar transporter-A) belonging to the glycoside-pentoside-hexuronide cation symporter transporter family is a poorly characterized member of this sugar transporter family. The gene was found to be often deleted from chromosome 1p in neuroblastoma cells and mutations of SLC45A1 have been associated with intellectual developmental disorders with neuropsychiatric features and autosomal recessive non syndromic intellectual disability. Given that glucose is the main brain fuel crossing the blood-brain barrier, the understanding of the properties of this sugar transporter is of great importance in a neurophysiological context.
The project aims to characterize the functional properties of SLC45A1, its cellular distribution and its physiological and pathophysiological role. In particular, we expressed the protein, fused with GFP, in HEK293 cells to define its subcellular localization. The fusion protein seems to be localized in intracellular vesicles of various sizes. Using a fluorescently labeled glucose analog, we also tested if SLC45A1 facilitates glucose uptake. Furthermore, we are mutating different putative intracellular motifs in an attempt to target the transporter to the plasma membrane in order to perform electrophysiological experiments to test for possible proton transport.
Moreover, using a SLC45A1 knockout mouse model we will determine the expression and the localization of the transcripts and the protein in different brain areas and neuronal cell types using qPCR and immunofluorescence and, using neuronal cultures dissociated from different areas of the KO mouse brain, we will study the physiological and pathophysiological role of SLC45A1 in brain metabolism.

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THE EFFECT OF GOLD IONS ON THE FORMATION OF BETALACTOGLOBULIN SUPRAMOLECULAR ASSEMBLIES

Anna Fricano [1], Giuseppe Sancataldo [1] and Valeria Vetri [1]

[1]Dipartimento di Fisica e Chimica, Università degli Studi di Palermo, V.le delle Scienze, Parco d'Orleans II, 90128, Palermo, Italy
anna.fricano@unipa.it

Biological materials like proteins and polypeptides exhibit unique self-assembly capabilities, making them valuable in creating ordered nanostructures such as amyloid fibrils. While these structures can be engineered to efficiently serve specific purposes in engineering materials and different technology applications. In order to develop hybrid materials with possible applications organic photovoltaic devices supported by the project Network 4 Energy Sustainable Transition (NEST), we investigated the supramolecular assembly of β-lactoglobulin (BLG) in the presence of gold. Gold is known for its high chemical stability and biocompatibility, can influence interactions between protein molecules, with the formation of protein-gold complexes. Moreover, gold is a good electrical conductor and also exhibits interesting optical properties which can be relevant for photovoltaic applications. Both the presence of protein nanofibers and of gold nanostructures have already been proved to increase the efficiency of organic solar cells. In general, hybrid gold-protein structures may offer several advantages and potential applications in this field.

β-Lactoglobulin can self-assemble into different macromolecular structure ranging from fibrils, microspheres or fractal gels and is considered a suitable building block for biomaterials. Fibrillar structures from this protein were used to template the spatial organization of titanium oxide nanostructures to improve photocatalytic efficiency.

We here report an experimental study of BLG supramolecular assembly at high temperature in the presence of gold ions in different conditions. This study is carried out using different spectroscopic techniques such as, fluorescence spectroscopy, circular dicroism (CD) and light scattering measurements.

BIOPHYSICAL AND PHARMACOLOGICAL ASPECTS OF FAMILIAL HEMIPLEGIC MIGRAINE 3 (FHM3) ARE RECOGNIZABLE IN DISSOCIATED CULTURES FROM CORTEX OF KNOCK-IN FHM3 MICE.

Raffaella Barbieri[1], Francesco Misurale[2], Susanna Alloisio[1,2], Tobias Freilinger[3], Michael Pusch1, Paola Gavazzo[1]

1 Biophysics Institute, CNR, Genoa, Italy
2 ETT Spa, Genoa, Italy
3 Department of Neurology, Klinikum Passau, Passau, Germany
paola.gavazzo@ibf.cnr.it

Familial hemiplegic migraine of type 3 (FHM3) is a severe form of migraine with aura caused by gain-of-function mutations in the *SCN1A* gene encoding the $Na_v1.1$ sodium channel. Even though several studies focused on FHM3, mechanisms underlying the disease are not fully understood, thus preventing a full comprehension of the disease causes. We recently elucidated molecular mechanisms by functionally characterizing *in vitro* all known FHM3 mutations, uncovering a unique major gain-of-function effect (GOF) underlying FHM3[1,2]. We confirmed the same defect also in neurons acutely dissociated from a knock-in mouse with the FHM3 L1649Q mutation[3], suggesting that the GOF is dominating over possible effects on protein stability. Furthermore, testing the late Na^+ current blocker (LSCB) GS967 we observed positive effects on the life-span of homozygous *Scn1a* L1649Q mice[3] and efficient correction *in vitro* of the GOF defects of FHM3 mutations[2]. The aim of the present study was testing the reliability of *in vitro* neuronal cultures in recapitulating biophysical and pharmacological properties of FHM3. Cultures were obtained from two knock-in mouse models, each one bearing a FHM3-related mutation, $Scn1a^{L1649Q}$ or $Scn1a^{L1670W}$, respectively. Double level of observation, i.e. recording from single cells and exploring neuronal network activity, was applied. Evaluation of kinetic parameters of the $Na_v1.1$ currents by patch-clamp pointed out sizeable differences between genotypes confirming an overall GOF effect of FHM3 mutants. Recording of neuronal spontaneous activity with a Multi–Electrode-Array (MEA) revealed a higher electrical activity of the cortical network and a reduced sensitivity to GS967 from homo- and heterozygous FHM3 mice compared to cultures from wild-type. The research adopts several strategies to reduce the number of animals employed, i.e. in vitro screening of therapeutic compounds, the use of neuronal cultures and of MEA devices which guarantee multiple registrations along network maturation from a single animal.

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AGAROSE-GUAR GUM-BASED EDIBLE COATING TO EXTEND THE STRAWBERRY SHELF-LIFE

C. Santalucia[1], A. Girgenti[1], D. Giacomazza[2], A. Pinto[3], S. Dallavalle[3], C. Dispenza[2,4], A. Quartieri[5], L. Campone[6,7], D. Nuzzo[1], P.L. San Biagio[2], T. Sanfilippo[8,9], M.R. Mangione[2], E. Muscolino[4], P. Picone[1]

[1] Istituto per la Ricerca e l'Innovazione Biomedica, CNR, Via U. La Malfa, 153 – 90146 Palermo (IT)
[2] Istituto di Biofisica (UOS Palermo), CNR, Via U. La Malfa, 153 – 90146 Palermo (IT)
[3] DeFENS, Università degli Studi di Milano, Via Celoria, 2 - 20133 Milano (IT)
[4] Dipartimento di Ingegneria, Università degli studi di Palermo, viale delle Scienze – 90128 Palermo (IT)
[5] Packtin srl, Via del Chionso 14/i – 42122 Reggio Emilia (IT)
[6] NBFC, National Biodiversity Future Center - 90133 Palermo (IT)
[7] Department of Biotechnology and Biosciences, University of Milano-Bicocca, Piazza della Scienza, 2 - 20126 Milano (IT)
[8] Ambulatorio di Nutrizione Clinica ASP Palermo, Via G. Cusmano 24, 90141 Palermo (IT)
[9] Anestesia e Rianimazione, Presidio Ospedaliero "S. Cimino", 90141 Termini Imerese, Palermo (IT)
daniela.giacomazza@cnr.it

Post-harvesting fruit and vegetable maintenance is a crucial problem for the horticulture and food market. Food loss means an economic damage at the retail and consumer levels.
Even when cold-stored, some fruits and vegetables have a very short life, thus many treatments were thought to preserve their texture, quality and nutritious value.
In the past, waxes have been used to preserve fruits from aging but the presence of carcinogenic chemical compounds present in the waxes has drastically reduced the use of these substances.
In the recent years, hydrocolloids have been used as coating systems to preserve fruit and vegetable integrity and freshness. Their high versatility and biocompatibility make them a perfect means to maintain fruit integrity.
In the present work we propose hydrogels obtained by different ratios of agarose (AG) and guar-gum (GG) acting as film coating to increase the shelf-life of strawberries.
SEM experiments investigated the spatial arrangement of the different blends.
A series of rheological measurements have been performed to detect the more suitable biopolymer mixture for its temperature behavior and ability to adhere to the strawberries, thanks to its viscosity and viscoelastic parameters obtained at different temperatures.
Our results indicated that the coating was able to protect strawberry, stored at 5 °C, up to 8 days from the treatment.

EXPLORING THE INTERPLAY OF GOLD NANOPARTICLE SIZE AND LIPID MEMBRANE CURVATURE IN MODULATING MEMBRANE FUSION

Beatrice Leonardini[1,3], Ester Canepa[1,2], Giorgia Brosio[1], Davide Bochicchio[1], Silvia Dante[3], Giulia Rossi[1], Annalisa Relini[1]

[1] Department of Physics, University of Genoa, 16146, Genoa, Italy
[2] Centre for BioNano Interactions, University College Dublin (UCD), D04 N2E5, Dublin, Ireland
[3] Materials Characterization Facility, Istituto Italiano di Tecnologia, 16163, Genoa, Italy

A wide range of fundamental cellular functions relies on lipid membrane fusion. Processes like those occurring in the biological milieu, can be developed using synthetic approaches aimed at designing controlled and selective membrane fusion in vitro. Specifically, membrane-embedded amphiphilic gold nanoparticles (AuNPs) can be employed as synthetic fusogens to induce vesicle fusion under physiological conditions1,2. Currently, we are focused on unraveling the interplay between lipid vesicle curvature and AuNPs size in the fusion process. The NP gold core curvature affects the conformational flexibility of amphiphilic ligands, which play a pivotal role in mediating interaction with the membrane during fusion3. In addition, membrane curvature is recognized as a crucial determinant in the fusion processes in vivo. Combining fluorescence spectroscopy assays and computational techniques, we studied the fusogenic properties of AuNPs of distinct sizes (2.4 nm and 4.8 nm - core diameter) interacting with large and small unilamellar vesicles containing a biologically relevant cholesterol percentage4. Our experimental and computational results revealed that small AuNPs promote vesicle fusion regardless of the membrane curvature. Conversely, large AuNPs did not exhibit fusogenic properties with low curvature membranes but suggest a tendency to induce fusion events with more curved membranes. Overall, these results indicate that, by fine-tuning these physical parameters, AuNPs are a promising synthetic fusion machinery for the development of tailored drug delivery tools.

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INVESTIGATION ON MMACHC-R161Q PATHOLOGICAL MUTANT FROM CBLC DISEASE, A RARE METABOLIC DISORDER OF VITAMIN B12 METABOLISM

Lisa Longo[1,2], Rita Carrotta[1], Maria Assunta Costa[1], Rosa Passantino[1], Maria Rosalia Mangione[1], Loredana Randazzo[1], Maria Grazia Ortore[3], Michela Bollati[1], Matteo De Rosa[1], Mario Milani[1], Adrian Velazquez Campoy[4], Marco Tutone[2], Vincenzo Martorana[1], Silvia Vilasi[1]

1 Istituto di Biofisica, Consiglio Nazionale delle Ricerche, Italy
2 Dipartimento Scienze e Tecnologie Biologiche Chimiche e Farmaceutiche, Università degli Studi di Palermo, Italy
3 Dipartimento di Scienze della Vita e dell'Ambiente, Università Politecnica delle Marche, Ancona, Italy
4 Institute for Biocomputation and Physics of Complex Systems (BIFI), Zaragoza, Spain

The cblC disease is a rare inborn disorder of the vitamin B12 (cobalamin, Cbl) metabolism characterized by combined methylmalonic aciduria and homocystinuria. The clinical consequences are devastating and, even when early treated with current therapies, the affected children manifest symptoms involving vision, growth, and learning[1]. The molecular genetic cause of the disease was found in the mutations of the gene coding for MMACHC, a 282 amino acid protein that transports and processes the various forms of Cbl[2]. Although the crystal structure of the wild-type protein is available[3,4], many molecular features of MMACHC physiopathology remain to be understood and a systematic study on the effect of each specific mutation on the resulting protein is still lacking. Here we present the biophysical characterization of wild type MMACHC and a variant, p.R161Q, resulting from the most common missense pathological mutation found in CblC patients. By using a biophysical approach, we investigated the stability of the two proteins and their ability to bind and transform the vitamin B12[5], and to assemble in a dimeric structure. Moreover, we evaluated whether drug-like molecules identified by computational methods, or non-specific stabilizers (osmolytes) could restore the functionality in MMACHC mutant. Overall, our results reveal how a biophysical approach based on the complementarity of computational and experimental methods can offer new insights in the study of the specific effects of the pathological cblC mutation and help prospecting new routes for the cblC treatment.

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A STRUCTURAL AND COMPUTATIONAL ANALYSIS OF *CAENORHABDITIS* ELEGANS CYCLIC NUCLEOTIDE-ACTIVATED CHANNEL TAX-4

Nicole Luchetti[1,2], Marco Lauricella[3], Velia Minicozzi[4], Grazia Cottone[5] and Letizia Chiodo[1]

[1]Dipartimento di Ingegneria, Università Campus Bio-Medico di Roma, Rome, Italy
[2]Center for Life Nano- & Neuro-Scienze, Istituto Italiano di Tecnologia, Rome, Italy
[3]Consiglio Nazionale delle Ricerche, Istituto per le Applicazioni del Calcolo IAC-CNR, Rome, Italy
[4]Dipartimento di Fisica & INFN, Università di Roma Tor Vergata, Rome, Italy
[5]Dipartimento di Fisica e Chimica Emilio-Segre', Università di Palermo, Palermo, Italy
e-mail: n.luchetti@unicampus.it

TAX-4 channel is a non-selective ligand-gated ion channel, expressed in several sensory neurons of *C. elegans* [1–3]. It is a nonselective intracellular cAMP- and cGMP-activated cation channel. It allows the permeation of both monovalent (Na^+, K^+) and divalent (Ca^{2+}, Mg^{2+}) ions, and also small charged molecules [4]. TAX-4 channel has a mammal ortholog, the cyclic nucleotide-gated channel subunit alpha 3 (CNGA3) channel, which is expressed in the cones [5,6]. These genes encode a member of the cyclic nucleotide-gated (CNG) cation channel protein family which is required for normal vision and olfactory signal transduction. Although their activity shows very little voltage dependence, CNG channels belong to the superfamily of voltage-gated ion channels [7,8], and they are composed by four identical subunits. In particular, CNG channels are responsible for intracellular Ca^{2+} dynamics in neurons and other sensory cells [6,9,10]. Mutations in these genes are associated with achromatopsia and color blindness [11–13] in mammals, while mutations in *C. elegans* are responsible for disfunction in thermosensation and chemosensation [14]. In this work we investigate the structural properties and ionic permeation of a wild-type CNG TAX-4 channel bound with cyclic guanosine monophosphate (cGMP) [10]. Starting from a recent atomic cryo-EM structure of the open state of the full-length *C. elegans* cGMP-activated channel TAX-4 [15], we perform standard all-atoms molecular dynamics simulations in a membrane/water system, to assess the stability of the open and functional conformation of the channel. We focus our attention on pore and binding site characteristics, analyzing the statistical behavior of the ligand in each binding site, characterizing the open state, and evaluating the permeation of sodium ions through the channel.

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HIGH SENSITIVITY AND SPECIFICITY MONITORING OF GASEOUS VOCS COUPLING A MULTIPASS GAS CELL SETUP WITH FTIR SPECTROSCOPY AND MACHINE LEARNING

Tiziana Mancini,[1] Annalisa D'Arco,[1] Maria Chiara Paolozzi,[2] Lorenzo Mosesso,[1] Rosanna Mosetti,[2] Salvatore Macis,[1] Augusto Marcelli,3 Francesco Radica,[4] Giovanna Tranfo,[5] Giancarlo Della Ventura[6] and Stefano Lupi[1,3]

1 Department of Physics, Sapienza University, Piazzale Aldo Moro 5, 00185 Rome, Italy;
2 Department of Basic and Applied Sciences for Engineering (SBAI), University of Rome 'La Sapienza', Via Scarpa 16, 00161 Rome, Italy;
3 National Institute for Nuclear Physics Laboratori Nazionali Frascati (INFN-LNF), Via E. Fermi 54, 00044 Frascati, Italy;
4 Department of Engineering and Geology, University Gabriele d'Annunzio Chieti-Pescara, Via dei Vestini, 66100 Chieti, Italy;
5 Department of Occupational and Environmental Medicine, Epidemiology and Hygiene, INAIL, Monte Porzio Catone, 00078 Rome, Italy;
6 Department of Science, University Rome Tre, Largo San Leonardo Murialdo 1, 00146 Rome, Italy;

The term Volatile Organic Compounds (VOCs) refers to a group of toxic organic chemical species able to evaporate at room temperature thanks to their high vapor pressure. They can easily spread in a close environment and are recognized to be dangerous for human health [1]. This is the main reason they need to be monitored in real time, in particular in working sites, where they are often found. Here, we will discuss the development of a new, and highly sensitive sensor system based on Infrared (IR) spectroscopy able to measure very low concentrations of various VOCs down to sub-ppm level and to discriminate among different chemical species, differently from currently used detectors, such as gas semiconductor sensors [2], gas chromatography, and/or mass spectrometry [3], photo- ionization detectors (PID), and so on. We will present the experimental setup, its performances, and the procedure to measure VOCs concentration from their IR absorbances [4]. The calibration curves for different VOCs are carried out, namely, styrene and several co-products (such as acetone, ethanol, isopropanol, benzene, toluene and xylene). The use of the calibration curves provides a reliable method to obtain sub-ppm_v concentrations well below the PID detection capability, as observed, in particular for styrene.

In order to mimic real conditions present in indoor environments, IR spectra of binary and ternary mixtures of the VOCs cited above are also measured and a data analysis procedure have been developed and optimized, obtaining excellent results in single VOC discrimination and in the extrapolation of their individual concentrations (in ppm), simply starting from their overall IR spectrum. Finally, a machine learning algorithm is being developed, optimizing the dataset processing, and evaluating the accuracy of different algorithms, with the final aim to provide the automatization of the single VOC detection starting from random mixtures. This work may constitute a comprehensive, very promising starting point for designing a portable device for the detection of gaseous pollutants in working environment, with a very high sensitivity and specificity, overcoming the limitations of currently used monitoring system.

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TOWARDS THE REAL-TIME OBSERVATION AND MANIPULATION OF BIOLOGICAL PROCESSES: FROM SINGLE MOLECULES TO WHOLE CELLS

Vincenzo Mascoli,[1] Emma Verver,[1] Andrea Candelli[1]

1 LUMICKS, Paalbergweg 3, 1105 AG Amsterdam, The Netherlands
v.mascoli@lumicks.com

Imagine you could directly see the location and dynamics of individual proteins binding to a single piece of DNA/RNA in real time. What if you could hold a single protein and manipulate its structure to interrogate its conformational landscape? What if you could push or pull on a cell and monitor its response in mechanical and biochemical terms?
Our unique platform combining high-end optical tweezers, fluorescence and label-free imaging, and microfluidics, makes that a reality! The LUMICKS C-Trap enables the real-time observation, control, and manipulation of biological processes at different scales, from individual molecules to whole cells. This combination of manipulation and visualization is essential to address complex biological mechanisms, not only at the highest resolution, but also in a dynamic fashion.
We will illustrate how this correlative technology changes the way we answer tough scientific questions in the fields DNA-binding enzymes, single-protein dynamics, membrane remodeling, and cell mechanobiology. Furthermore, we show that advances in hybrid single-molecule methods can be turned into an easy-to-use and stable instrument that has the ability to open up new avenues in many research areas.

FUNCTIONAL CHARACTERIZATION OF THE RECOMBINANT HUMAN RIBOFLAVIN TRANSPORTER 2 (SLC52A2)

Irene Mazza[1,2], Lara Console[2], Maria Tolomeo[3], Maria Barile[3], Cesare Indiveri[2,4]

[1]Department DiBEST (Biologia, Ecologia, Scienze della Terra) Unit of Biochemistry and Molecular Biotechnology, University of Calabria, Arcavacata di Rende, Italy
[2]Department of Biosciences, Biotechnology, and Biopharmaceutics, University of Bari, Bari, Italy
[3]CNR Institute of Biomembranes, Bioenergetics and Molecular Biotechnologies (IBIOM), Bari, Italy
irene.mazza@ibf.cnr.it

Riboflavin is a water-soluble vitamin essential for cell viability. Indeed, it is the precursor of FMN and FAD, which are crucial for metabolizing carbohydrates, amino acids, and lipids. Since higher organisms, including humans, have lost the ability to synthesize Riboflavin, this vitamin must be absorbed from food. Its adsorption and tissue distribution occur via three specific transporters belonging to the SLC52 family: SLC52A1, SLC52A2, and SLC52A3.
In particular, several polymorphisms of the SLC52A2 gene have been associated with a rare neurological disorder known as Riboflavin Transporter Deficiency (RTD). A combined approach that includes the reconstitution of transport protein into proteoliposomes and bioinformatic analyses has been adopted to perform the functional characterization of SLC52A2. Therefore, human transport protein has been overexpressed in E. coli, purified, and then reconstituted into proteoliposomes to measure the transport activity following the [^{3}H]Riboflavin accumulation into the vesicles.
SLC52A2 showed a Km of 0.26 ± 0.07 μM and is inhibited by lumiflavin and FMN. On the contrary, FAD is unable to inhibit the transporter. Experimental data on transport inhibition correlate with the docking analysis. The described approach is also suitable for investigating the SLC52A2 variants associated with human pathologies.

CHARACTERIZING CU(I)- AND CU(II)-AMYLOID BETA PEPTIDE COMPLEXES: A COMPUTATIONAL AND EXPERIMENTAL APPROACH

S. Alleva[1] , A. Muroni[1], E. De Santis[1,2,3], R. Bean[4], V. Minicozzi[1] and F. Stellato [1]

[1]Department of Physics and INFN, University of Rome – Tor Vergata, Roma, 00133, Italy
[2]Department of Chemistry – BMC, Uppsala University, Box 576, SE-751 23 Uppsala, Sweden
[3]Department of Physics and Astronomy, Uppsala University, Box 516, SE-751 20 Uppsala, Sweden
[4]European XFEL GmbH, Holzkoppel 4, 22869, Schenefeld, Germany
velia.minicozzi@roma2.infn.it

The aetiology of the so-called *protein conformational disorders,* like Alzheimer's and Parkinson's diseases, is in general related to an unfolded protein: the unfolded or misfolded protein loses its functionality and can also become toxic. One of the many factors influencing the *misfolding* process, is metal ions presence [1], in particular Cu, Zn and Fe. Moreover, Cu ions bound to the amyloid-β (Aβ) peptide represent a source of oxidative stress in the context of Alzheimer's disease because of their redox activity. In order to explain the efficient redox cycling between Cu(II)-Aβ (distorted square-pyramidal) and Cu(I)-Aβ (digonal) resting states, the existence of a low-populated "in-between" state, prone to bind Cu in both oxidation states, has been postulated [2].

Here, we describe a multi-scale computational pipeline aimed at reproducing the resting and the "in-between" states of Cu-Aβ and at better interpreting X-ray absorption (XAS) spectra and Free Electron Laser coherent imaging data.

We start by performing classical Molecular Dynamics (MD) simulations of both Cu(I)- and Cu(II)-Aβ complexes, modelling the metal ions with dummy-atoms [3, 4, 5]. The configurations coming out from MD will then be validated with deep-learning methods [6] and quantum mechanical calculations. Finally, theoretical X-ray absorption spectra will be calculated to compare our numerical results with experimental observables [7, 8, 9].

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A SIZE-DEPENDENT DIVISION STRATEGY ACCOUNTS FOR LEUKEMIA CELL SIZE HETEROGENEITY

Mattia Miotto[1] *Simone Scalise*[1], *Marco Leonetti*[2], *Giancarlo Ruocco*[1,3], *Giovanna Peruzzi*[1], *Giorgio Gosti*[2]

[1]Center for Life Nano & Neuro Science, Istituto Italiano di Tecnologia, Rome, Italy
[2]Soft and Living Matter Laboratory, Consiglio Nazionale delle Ricerche, Rome, Italy
[3]Department of Physics, Sapienza University of Rome, Rome, Italy
mattia.miotto@iit.it

Cell size exhibits a huge variability across different kinds of cells. However, cells of isogenic populations have a well defined typical size, preserved despite the complex and noisy machinery of cellular processes. How this is achieved is a question that remains still largely unanswered. From a reductionist cell dynamics point of view, the strategies allowing size homeostasis are roughly grouped into three classes, i.e. timer, sizer or adder, empirically distinguishable given the relationship between cell size at birth and at division. Here, we will show how it is possible to infer the growth regime and division strategy using flow-cytometric data of marked cells [1] and forward side scattering as a proxy for cell size. Comparing the experimental data with the prediction of a minimal mathematical model, we found that (i) leukemia Jurkat T-cells in standard growth conditions follow a sizer-like strategy, with (ii) division times following an Erlang distribution given by the sum of exponentially-distributed times. Moreover, our work shows that (iii) the dynamics of the size distribution is reproduced by a minimal model where both cell growth and division rates depends on powers of the cell size [2].

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*contributed equally.

SUPER-RESOLUTION MICROSCOPY HIGHLIGHTS CELLS BEHAVIOUR ON POLYMER NANOFIBERS

Rebecca Mordini[1] *Fabio Lineu Favrin*[1], *Lorenzo Lavista*[1,2], *Nicola Puccini*[1], *Chiara Cavallini*[2], *Andrea Camposeo2, Luana Persano*[2], *Francesca Cella Zanacchi*[1,3,4] *and Dario Pisignano,*[1,2,4]

[1]Università di Pisa, Dipartimento di Fisica E. Fermi, Largo B. Pontecorvo 3, 56127 Pisa, Italy
[2]NEST, Istituto Nanoscienze-CNR and Scuola Normale Superiore, Piazza S. Silvestro 12, 56127 Pisa, Italy
[3]Nanoscopy and NIC@IIT, Istituto Italiano di Tecnologia, 16152 Genova, Italy
[4]Centro per l'Integrazione della Strumentazione dell'Università di Pisa (CISUP), Università di Pisa, 56127 Pisa, Italy
r.mordini@studenti.unipi.it

Nanofiber substrates (NS) have been widely used as functional scaffolds for tissue engineering and regenerative medicine, thanks to their ability to mimic features of the extracellular matrix, including fibrous morphology and mechanical properties. Recent studies have shown that NS can be engineered in order to send signals to the cellular environment[1]. Consequently, cells respond exhibiting transformations in behaviour, which could be accompanied by modulation in chromatin structure inside the nucleus and by precise regulation of gene expression[2,3].

However, little is still known about how these modulations occur. The main goal of this work is to understand how cells rearrange in response to mechanical cues, through single-molecule localization microscopy. We have produced NS made of poly(D,L-lactide-*co*-glycolide) (PLGA), a biocompatible polymer largely employed in tissue engineering and regenerative medicine[4], by means of the electrospinning technique. Using Stochastic Optical Reconstruction Microscopy (STORM) then allows one to gain cell information at the molecular scale level[5].

The deeper understanding of the cellular response to nanofiber signals can pave the way for more precise and effective scaffold design in tissue engineering. Additionally, it can contribute to the development of advanced regenerative medicine strategies that harness the unique properties of NS to promote controlled and personalized tissue regeneration and repair.

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TARGETING THE RHOA-ROCK SIGNALING PATHWAY FOR TREATMENT OF EARLY-STAGE MELANOMA TUMORS

Miguel Moreira,[1] Nicoletta Brindani,[1] Maria Antonietta La Serra,[1] Enrico Bergamaschi,[1] Isabella Maria Acquistapace,[1] Marco De Vivo[1]

[1] Molecular Modeling and Drug Discovery – Istituto Italiano di Tecnologia, Via Morego 30, 16163 Genova, Italy
miguel.cardoso@iit.it

Melanoma accounts for 80% of skin cancer-related deaths[1]. The major form of treatment is surgical resection, but where such an approach is not possible or feasible, immunotherapy or targeted therapies are key. Such therapies include BRAF inhibitors such as verumafenib[2,3] and immune checkpoint inhibitors (e.g. pembrolizumab and ipilimumab). However, side effects and acquired resistance are key limitations to overcome[4]. Combinatorial approaches employing multi anticancer agents (radiation, chemotherapy, and surgical excision) have also been used. Despite all these approaches, a significant decrease in melanoma-related mortality has not been observed[5]. Together with the side effects, the resistance, and the toxicity of current therapeutic strategies, this underlies a dire and urgent need for new therapeutic approaches against melanoma[5,6].

RHOA (Ras homologous A) belongs to the family of RHO GTPases (generally classified under the umbrella of the undruggable targets) and it is a pivotal intramolecular signaling switch involved in the regulation of cytoskeleton dynamics, cell migration and differentiation[7], processes deeply altered in neoplastic states[7,8]. Indeed, RHOA is overexpressed and/or hyper-activated in a plethora of cancers[7,9]. Modulation/inhibition of RHOA represents an emerging and promising strategy to target a myriad of cancers, including melanoma.

Our work focuses on the modulation/inhibition of the interaction between RHOA and its downstream effector ROCK1 (RHO associated coiled-coil kinase) that ultimately regulates cytoskeletal dynamics critical for melanoma progression[10]. Our approach relies on the targeting of a pocket present in the active conformation of RHOA, which is crucial for the interaction with ROCK1.

We produced and purified recombinant RHOA and ROCK1 and characterized them using a plethora of biophysical and biochemical methods such as circular dichroism (CD) spectroscopy, mass spectroscopy (MS), dynamic light scattering (DLS), and thermofluor assays. Using techniques such as intrinsic fluorescence and biolayer interferometry, we characterized the binding between RHOA and ROCK1. Furthermore, using a library of small molecules, we attempted to characterize small molecules binding to RHOA using intrinsic fluorescence, microscale thermophoresis and biolayer interferometry. Subsequently, we characterized the capacity to inhibit or disrupt the interaction between RHOA and ROCK1 using nuclear magnetic resonance (specifically waterLOGSY experiments). Protein crystallography is also exploited to obtain three-dimensional structures of RHOA in complex with the best ligands to decipher key interactions and guide the design of new small molecules using medicinal chemistry approaches.

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SUPER-RESOLUTION STORM MICROSCOPY OF PRINTED PROTEINS

Alberto Morgantini[1], Fabio Lineu Favrin[1], Francesca Matino[2], Luana Persano[2], Francesca Cella Zanacchi[1,3,4] and Dario Pisignano[1,2,3]

[1]Dipartiment of Physics, University of Pisa, Largo Bruno Pontecorvo 3, 56127, Pisa, Italy
[2]NEST, Istituto Nanoscienze-CNR, 56126 Pisa, Italy
[3]Center for Instrumental Sharing of the University of Pisa (CISUP), Pisa, Italy
[4]Nanoscopy and NIC@IIT, Istituto Italiano di Tecnologia, 16152 Genoa, Italy

Microcontact printing (μCP) is employed for implementing molecular substrates for various purposes: from biosensor design to scaffolding for cell growth. This is an efficient technique for pattern transfer, allowing for the replication of a specific geometry onto a substrate through the deposition of monolayers of specific substances[1]. In this context, the goal of this study is to characterize physiologically self-assembled protein patterns using super-resolution fluorescence microscopy[2]. Stochastic Optical Reconstruction Microscopy (STORM) is a super-resolution fluorescence microscopy technique belonging to the family of single molecule localization microscopy (SMLM)[3]. These techniques overcome the diffraction limit inherent in traditional microscopy, pushing the image resolution to the order of tens of nanometers, thus providing an optimal tool for nanoscale characterization of self-assembled protein.
After μCP, nanometric protein structures are measured with atomic force microscopy (AFM) and imaged by STORM microscopy upon optimized an immunostaining protocol for super-resolution imaging. Numerous tools already exist for managing the spatial point patterns of localized fluorophores provided as STORM output[4]. Still, most of them are focused on the recognition and characterization of cluster structures, while only a few focus on the reconstruction of diffuse structures. In this work, we aim at optimizing the analysis of the large protein structures comparing analysis tools based on spatial correlation, network analysis[5] and localization density analysis[6]. These tools can be exploited for several different applications, such as pattern and subcellular structure recognition and other biomedical purposes.
We acknowledge the support of the European Union by the Next Generation EU project ECS00000017 'Ecosistema dell'Innovazione' Tuscany Health Ecosystem (THE, PNRR, Spoke 4: Nanotechnologies for diagnosis and therapy) and CISUP Center for Instrumental Sharing of the University of Pisa.

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INFRARED SPECTROSCOPY INVESTIGATION OF SARS-COV-2 SPIKE PROTEIN DOMAINS

Rosanna Mosetti,[1] Annalisa D'Arco,[1] Tiziana Mancini,[1] Salvatore Macis,[1] Marta Di Fabrizio,[2] Velia Minicozzi,[3] Giancarlo Della Ventura,[4,5] Augusto Marcelli,[5] Nicole Luchetti [6,7] and Stefano Lupi [1,8]

[1] Department of Physics, University of Rome 'La Sapienza', P.le A. Moro 2, 00185 Rome, Italy.
[2] Laboratory of Biological Electron Microscopy, School of Basic Sciences, Institute of Physics, EPFL & Department of Fundamental Microbiology, Faculty of Biology and Medicine, UNIL, 1015 Lausanne, Switzerland.
[3] Department of Physics, University of Rome Tor Vergata, Via della Ricerca Scientifica, 100133 Rome, Italy.
[4] Department of Science, University Rome Tre, Largo San Leonardo Murialdo 1, 00146 Rome, Italy.
[5] Laboratori Nazionali Frascati, National Institute for Nuclear Physics (INFN-LNF), Via E. Fermi 54, 00044 Frascati, Italy.
[6] Engineering Department, University Campus Bio-Medico of Rome, Via Alvaro del Portillo 21, 00128, Rome, Italy
[7] Centre for Life Nano- and Neuro-Science, Italian Institute of Technology, Viale ReginaElena 291, 00161, Rome, Italy.
[8] National Institute for Nuclear Physics Section Rome1, P.le A. Moro 2, 00185 Rome, Italy.

Coronaviruses are characterized by Spike (S) glycoproteins, which are the largest structural membrane proteins and the first involved in the anchoring of the host receptor angiotensin-converting enzyme 2 (ACE2) through the receptor binding domain (RBD). The SARS-CoV-2 S protein is composed by two subunits: S1, responsible for recognition/anchoring to the host receptor ACE2 and containing the RBD; S2, responsible for the membrane fusion [1,2]. This protein secondary structure is of great interest for shedding light on various aspects, from functionality to pathogenesis, finally to spectral fingerprint for the design of optical biosensors. The aim of this work is the characterization of the whole monomeric S protein, its domains and three variants at a serological pH (7.4) by measuring their amide I infrared absorption bands through Attenuated Total Reflectance Infrared spectroscopy (ATR-IR), one of the main and suitable techniques used in the secondary structure analysis of proteins [3,4,5].

Being the S1 involved in the viral process, it is useful to study its secondary structure alterations induced to chemical/physical environmental modifications and/or to mutations in the SARS-CoV-2 variants. For this reason, we performed a study on the S1 (variant of March 2020) subunit under pH [4] and temperature variations, observing how the S1 protein adapts its secondary structure to different inhospitable surroundings. The high stable configuration is present at endosomal pH 5.5 and at temperature of 30°C, proving that the protein evolution modifications yield the protein more performant [4]. Furthermore, we perform a comparison of IR spectroscopic and Circular Dichroism (CD) secondary structure analyses of the S1 monomer of three SARS-CoV-2 variants, showing how IR technique allows their discrimination.

Experimental data in combination with MultiFOLD predictions, Define Secondary Structure of Proteins (DSSP) web server (DSSP) and Gravy value calculations, provide a comprehensive understanding of proteins' domains in terms of their secondary structure content, conformational order and interaction with the solvent.

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HUMAN CYTOCHROME C NATURAL VARIANTS: STUDYING THE MEMBRANE BINDING PROPERTIES OF G41S AND Y48H BY FLUORESCENCE ENERGY TRANSFER AND MOLECULAR DYNAMICS

Alessia Muroni[1], Velia Minicozzi[1], Maria Cristina Piro[2], Federica Sinibaldi[2], Giampiero Mei[2], Almerinda Di Venere[2]

[1] Department of Physics, Tor Vergata University of Rome, Via della Ricerca Scientifica 1, 00133 Rome, Italy
[2] Department of Experimental Medicine and Surgery, Tor Vergata University of Rome, Via Montpellier 1, 00133 Rome, Italy

In recent years, numerous investigations into naturally occurring mutations within cytochrome c (cyt-c) have underscored its role in various genetic disorders. Cyt-c is a pivotal protein situated within the intermembrane space of mitochondria in eukaryotic cells, playing a fundamental role in both cellular respiration and apoptosis. It comprises a single polypeptide chain containing a prosthetic group known as heme. During the process of cellular respiration, cyt-c facilitates the transfer of electrons from complex III to complex IV within the electron transport chain, a function made possible by its heme group. During apoptosis, various cellular stress signals trigger the release of cyt-c from the mitochondria into the cytoplasm. In the cytoplasm, cyt-c binds with other proteins to form the apoptosome, which activates caspase enzymes. Activation of caspases initiates a cascade of events leading to cell death, helping to remove damaged or unwanted cells from the body. Among the naturally occurring mutations in cyt-c, two noteworthy substitutions have been observed: glycine 41 replaced by serine (G41S) and tyrosine 48 replaced by histidine (Y48H). These mutations have been detected in individuals afflicted with thrombocytopenia, a medical condition marked by a reduced platelet count in the bloodstream, often leading to symptoms like skin bleeding and bruising. Different computational studies on these mutants have highlighted their greater involvement in the reduction of platelet formation and the increase in peroxidase activity during the apoptosis process [1, 2]. These studies, however, have primarily focused on analyzing structural differences between cyt-wt and mutated cytochromes in aqueous solution. The purpose of our research is to study for the first time the interaction between the different types of cytochromes (wt, G41S and Y48H) and cellular membranes in order to better understand the role of natural occurring mutations on the developement of several genetic diseases. These simulations will be fundamental to explain the results of different experiments in which it was demonstrated that the mutant cytochromes bind more weakly to the membranes compared to the cyt-wt. In the initial phase of our study, Molecular Dynamics (MD) simulations were conducted in the "isothermal-isobaric" ensemble (NPT) using systems comprising a POPC membrane and a cyt-c molecule. Subsequently, the investigation progressed to explore the interactions between the wt cytochrome and the mutant cytochromes within a more realistic membrane composed of POPC-cardiolipin (50%-50%). MD simulations were performed on three systems for each cytochrome (wt, G41S, and Y48H) with a POPC membrane and three systems with a cardiolipin-based membrane.

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CONCANAVALIN A DELIVERS A PHOTOACTIVE PROTEIN TO THE BACTERIAL WALL

Andrea Mussini[1,2] Pietro Delcanale,1 Melissa Berni,[3] Stefano Pongolini,[3] Mireia Jordà-Redondo,[2] Montserrat Agut,[2] Peter J. Steinbach,[4] Santi Nonell,[2] Stefania Abbruzzetti,[1] Cristiano Viappiani[1]

[1] Dipartimento di Scienze Matematiche, Fisiche e Informatiche, Università di Parma, Parco Area delle Scienze 7A, 43124, Parma, Italy
[2] Institut Químic de Sarrià, Universitat Ramon Llull, Via Augusta 390, 08017, Barcelona, Spain
[3] Risk Analysis and Genomic Epidemiology Unit, Istituto Zooprofilattico Sperimentale della Lombardia e dell'Emilia-Romagna, Strada dei Mercati, 13/A, 43126, Parma, Italy
[4] Bioinformatics and Computational Biosciences Branch, National Institute of Allergy and Infectious Diseases, National Institutes of Health, Bethesda, MD 20892, USA
andrea.mussini@unipr.it

Modular supramolecular complexes, where different proteins are assembled to gather targeting capability and photofunctional properties within the same structures, are of special interest for bacterial photodynamic inactivation, given their inherent biocompatibility and flexibility. We have recently proposed one such structure, exploiting the tetrameric bacterial protein streptavidin as the main building block, to target *S. aureus* protein A.[1]
To expand the palette of targets, we have linked biotinylated Concanavalin A, a sugar binding protein, to a methylene blue labelled streptavidin. By applying a combination of spectroscopy and microscopy, we demonstrate binding of Concanavalin A to the wall of Gram-positive *S. aureus* and Gram-negative *E. coli.* Photoinactivation is observed for both bacterial strains in the low micromolar range, although the moderate affinity for the molecular targets, and the low singlet oxygen yields limit the overall efficiency. Finally, we propose a novel approach to the analysis of autocorrelation traces, based on the Maximum Entropy Method, which proves particularly useful to describe signals measured for diffusing systems heterogenous in size, such as fluorescent species bound to bacteria.

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SPECTROSCOPIC METHODS ENABLING THE CIRCULAR ECONOMY: INVESTIGATING NOVEL APPLICATIONS FOR DAIRY AND TEXTILE BY-PRODUCTS FROM ITALIAN SUPPLY CHAINS

Antonino Natalello,[1] Diletta Ami,[1] Ivana Dragojlov,[2] Greta Bianchi,[1] Barbara Sciandrone,[1] Alex Pessina,[1] Alessandro Palmioli,[1] Samuele Signorelli,[1] Cristina Airoldi,[1] Maria Elena Regonesi,[1] Luca Brambilla,[1] Marina Lotti,[1] Marco Mangiagalli[1], Stefania Brocca[1], Simone Vesentini,[2] Laura Cipolla[1]

[1] Department of Biotechnology and Biosciences, University of Milano-Bicocca, Piazza della Scienza 2, 20126 Milan, Italy.
[2]Department of Electronics, Information and Bioengineering, Politecnico di Milano (Italy), Piazza Leonardo da Vinci 32, Milano, 20133, Italy.
antonino.natalello@unimib.it

Dairy and textile production systems, though distinct in product categories, share a commonality in their highly linear value chains, leading to the generation of unutilized or underutilized by-products that pose environmental risks when discarded.

Here, we will introduce a circular chain model dedicated to repurposing by-products from both the dairy and textile industries, for the production of protein and protein-based biomaterials. Emphasizing the crucial role of biophysical approaches, particularly infrared (IR) and circular dichroism (CD) spectroscopies, we illustrate their application in monitoring the biological processes of interest.

Fourier transform infrared (FTIR) microscopy was applied on intact *Escherichia coli* cells to investigate cellular effects of recombinant protein expression induced by cheese whey permeate (CWP), a secondary by-product of the dairy industry. Overall, micro-FTIR results indicate the effectiveness of CWP as an inducer of recombinant expression and the role of CWP micronutrients in alleviating lipid membrane perturbations associated with heterologous expression[1].

In the context of textile chains, we propose the utilization of sericin protein, a by-product in silk yarn production. Our focus lies in optimizing electrospinning conditions for sericin from different sources and degumming approaches to obtain innovative biomaterials. Combining FTIR and CD spectroscopies along with scanning electron microscopy, we found a correlation between the conditions of biomaterial preparation, internal protein structures, and the morphology of the resulting biomaterials.

In conclusion, this research highlights the importance of spectroscopic methods, their utility in studying protein-based biomaterials, and their role in designing sustainable practices in the dairy and textile industries.

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IMAGING CYTOSKELETAL NETWORKS: FROM QUANTITATIVE FLUORESCENCE TO AI-ASSISTED SUPER-RESOLUTION MICROSCOPY

Benedetta Noferi[1], Vittorio Parenti[1], Claudia Martini[2,3,4], Martina de Felice[1,2], Francesca del Debbio[2], Eleonora da Pozzo[2,3,4], Raffaella Magrassi[5,6], Simone Lossano[1,7], Francesca Lizzi[7], Camilla Scapicchio[7], Alessandra Retico[7], Simone Capaccioli[1,3,4] and Francesca Cella Zanacchi[1,3,5]

[1]Department of Physics E. Fermi, University of Pisa, Largo Bruno Pontecorvo, 56127 Pisa, Italy
[2] Department of Pharmacy, University of Pisa, Via Bonanno Pisano, 12, 56126 Pisa, Italy
[3]Center for Instrumental Sharing of the University of Pisa (CISUP), Pisa, Italy
[4] Centro Pisano Multidisciplinare Sulla Ricerca e Implementazione Clinica della FLASH Radiotherapy (CPFR), University of Pisa,
Presidio Santa Chiara, ed.18 Via Roma 67, 56126 Pisa, Italy
[5] Nanoscopy and NIC@IIT, Istituto Italiano di Tecnologia, 16152 Genoa, Italy
[6] Istituto di Biofisica, Research National Council (CNR) Genoa, Italy
[7] National Institute for Nuclear Physics (INFN) – Pisa Section, Via Filippo Buonarroti, 3, 56127 Pisa, Italy

In recent years, there has been a rapid evolution in fluorescence microscopy techniques, transforming them into powerful tools for studying biological structures and nanoscale bio-molecular arrangements. Each fluorescence microscopy technique possesses distinct advantages and limitations, allowing the investigation of specific aspects of cellular cytoskeleton networks[1]. While conventional methods like epifluorescence microscopy or more specialized approaches, such as total internal reflection (TIRF) microscopy, may offer sufficient quantitative data, a true breakthrough lies in overcoming the diffraction limit through Single Molecule Localization Microscopy (SMLM)[2]. In particular, STORM (STochastic Optical Resolution Microscopy) represents a powerful tool featuring an effective resolution below 20 nm. However, this revolutionary approach faces limitations, primarily linked to its limited temporal resolution and to the pointillistic nature of the output, often driven by a partial fluorophores labeling efficiency. In this context, Artificial Intelligence (AI) approaches have gained prominence, offering super-resolution images directly from diffraction-limited inputs[3]. In our work, we designed a multiscale approach that combines epifluorescence, highly-inclined illumination microscopy, and AI-assisted super-resolution to fully characterize cytoskeletal proteins' organization at the sub-cellular level. We adapted a Generative Adversarial Network (GAN) to reconstruct super-resolution images of filamentous structures, such as microtubules, directly from a low-resolution input as widefield microscopy. Moreover, our research showcased an enhanced ability to obtain more accurate estimations of filament anisotropy compared to conventional STORM imaging. We thus developed a tool of undeniable usefulness alongside fluorescence microscopy for studying the spatial arrangement of cytoskeletal structures. We used this tool to study the *in vitro* effects induced by an innovative radiotherapy technique, Flash radiotherapy. Specifically, we explored molecular rearrangements at the cytoskeletal level, comparing the novel Flash RT with conventional radiotherapy techniques. Despite FLASH radiotherapy demonstrating efficacy in controlling tumor growth while significantly reducing damage to healthy tissues, its underlying mechanism remains largely unclear[4]. In this context, our work provides a valuable tool to investigate the "FLASH effect" and contributes to shedding new light on the molecular responses induced by different irradiation modalities. Acknowledgements: The authors thank the "Centro per l'Integrazione della Strumentazione dell'Università di Pisa (CISUP), the "Centro Pisano Multidisciplinare sulla Ricerca e Implementazione Clinica della Flash Radiotherapy (CPFR)" and Fondazione Pisa for funding the project "prog. n. 134/2021". The research was partly supported by the project "Piano Nazionale di Ripresa e Resilienza (PNRR), Missione 4, Componente 2, Ecosistemi dell'Innovazione—Tuscany Health Ecosystem (THE), Spoke 1 "Advanced Radiotherapies and Diagnostics in Oncology"—CUP I53C22000780001" and the project. PNRR - M4C2 - Investimento 1.3, Partenariato Esteso PE00000013 - "FAIR - Future Artificial Intelligence Research" - Spoke 8 "Pervasive AI", funded by the European Commission under the NextGeneration EU programme.

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A NANOSCALE EXPLORATION OF THE AGGREGATION OF PARTIALLY LABELLED POLYPEPTIDES VIA AFM-STED CORRELATIVE MICROSCOPY.

Davide Odino,[1] Elena Angeli,[1] Annalisa Relini,[1] Paolo Bianchini,[2] Alberto Diaspro,[1,2] and Claudio Canale[1]

[1]Department of Physics, University of Genoa, Genoa, Italy; [2]Nanoscopy, CHT Erzelli, Istituto Italiano di Tecnologia, Genoa, Italy
davide.odino@edu.unige.it

Aggregation leading to the formation of highly organized amyloid fibrils, rich in β-sheets, is a common feature of polypeptide chains[1]. The formation and deposition of amyloid aggregates in the body is frequently associated with degenerative diseases. Therefore, a detailed understanding of the molecular mechanisms of protein aggregation is crucial to counteract protein misfolding diseases. From this perspective, AFM-STED correlative microscopy has proven to be a powerful investigative tool capable of obtaining details on biological processes at the previously inaccessible molecular level. In this research, we explore the aggregation of bovine pancreatic insulin using two different fluorescent markers, ATTO647-N and ATTO594.

We followed standard protocols to promote fibrillation and to label peptides. using two distinct fluorophores functionalized with an NHS ester group. We considered dye-to-protein ratios commonly adopted and reported in the literature[2]. The results highlight several aspects: first, in line with previous research[3], only a fraction of the fibrillar aggregates was fluorescent, demonstrating that the labelled peptides only contribute to the formation of a subset of fibrils. Furthermore, a significant number of fibrils show a single fluorescent species, supporting the idea that the presence of two different dyes induce selectivity in the aggregation process. In summary, the aggregation produces four types of aggregates: unlabeled fibrils, ATTO647-N labeled fibrils, ATTO594 labeled fibrils, and two colours' fibrils. The approach outlined in this study highlights the coexistence of different aggregation mechanisms: heterogeneity and selectivity in the amyloid aggregation process is induced by fluorophores.The next step of our research aims to find structural differences between the four categories of fibrils above mentioned.

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A QUANTITATIVE METHOD BY SUPER-RESOLUTION MICROSCOPY TO CHARACTERIZE EARLY DNA-DAMAGE AND REPAIR INDUCED BY FLASH RADIOTHERAPY

Vittorio Parenti,[1] Benedetta Noferi,[1] Martina De Felice,[1,2] Margherita Maffei, [5] Gaia Scabia, [5] Fabio di Martino,[4,7] Damiano del Sarto,[4,3] Fabiola Paiar,[4,8] Simone Capaccioli,[1,3,4] Francesca Cella Zanacchi[1,3,6]

[1] Department of Physics E.Fermi, University of Pisa, Pisa, Italy.
[2] Department of Pharmacy, University of Pisa, Pisa, Italy.
[3] Center for Instrumental Sharing of the University of Pisa, Pisa, Italy (CISUP).
[4] Multidisciplinary Pisan Center on Research and Clinical Implementation of Flash Radiotherapy, Pisa, Italy (CPFR).
[5] National Research Council - Institute of Clinical Physiology, Pisa, Italy (CNR-IFC).
[6] Nanoscopy and NIC@IIT, Istituto Italiano di Tecnologia, 16152 Genoa, Italy.
[7] Health physics unit, Santa Chiara Pisa hospital, Pisa, Italy
[8] Department of translation research and new technologies in medicine and surgery, University of Pisa, Pisa, Italy.

In the exploration of cutting-edge radiotherapy techniques, FLASH radiotherapy stands out as a revolutionary approach diverging from conventional methods (CONV-RT). Unlike its traditional counterpart, FLASH delivers a singular, ultra-high dose at rapid rates, boasting comparable efficacy in tumor growth control while significantly mitigating harm to healthy tissues - a phenomenon known as the "sparing effect" [1,2]. However, the understanding of this effect requires further investigation, given the lingering ambiguity surrounding its mechanisms [3]. In this context, advanced biophysical tools, such as super-resolution microscopy, may be utilized for novel insights that are scarcely accessible through commonly employed approaches in standard radiobiological studies. The study employed the capabilities of super-resolution single-molecule localization microscopy to quantitatively analyze the impact of irradiation on the distribution of proteins involved in the DNA damage and repair process. Specifically, STORM (STochastic Optical Resolution Microscopy), a state- of-the-art fluorescence Super Resolution imaging technique with sub-20 nm resolution, was used. In addition, a quantitative method based on clustering analysis was optimized to characterize gammaH2AX histones, which are fundamental proteins involved in the early response to DNA damage and repair. As a member of the Single- Molecule Localization Microscopy (SMLM) family, STORM facilitated the study of biological structures and biomolecular arrangements at the nanoscale. Furthermore its integration with clustering analysis provides a valuable tool to transform the imaging technique into a real quantitative tool, able to perform quantitative studies. As a case study, the optimized method was applied to characterize irradiation-induced DNA damage for Familial Partial Lipodystrophy (FPLD), a rare genetic disorder with peculiar behavior under irradiation [4]. The study focuses on the effects induced by different radiotherapeutic conditions, Conventional-RT and FLASH-RT, analyzing cellular responses in mutated cells from FPLD- patients compared to healthy samples. This entails a quantitative characterization of repair foci, which are localized concentrations of proteins involved in the DNA repair process. The signal obtained directly correlates with the number of protein recruited to the damaged site. Subsequently, density-based clustering algorithms are employed to determine protein quantities within the clusters obtained, facilitating the analysis of phosphorylated histones and their densities at the repair sites. Additionally, the analysis extends to variations in response under different radiation doses and the divergent behaviour of cells carrying the FPLD mutation. This research represents a further advancement in the in vitro characterization of the FLASH sparing effect through state-of-the-art biophysical and imaging tools. Acknowledgements: The authors thank the “Centro per l'Integrazione della Strumentazione dell'Università di Pisa (CISUP)”, the “Centro Pisano Multidisciplinare sulla Ricerca e Implementazione Clinica della Flash Radiotherapy (CPFR)”.

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CORRELATIVE RAMAN-BRILLOUIN SPECTROSCOPY: INVESTIGATING FIXATION EFFECTS ON CELLS

Passeri, A.A.[1], Argentati, C.[2] , Morena, F.[2] , Neri, I.[1] , Bonacci, F. [1], Fioretto, D.[1] , Vassalli M.[4], Martino, S.2, Mattarelli, M.[1,] Caponi, S.[3]

1 Department of Physics e Geology, University of Perugia, I-06100 Perugia, Italy
2 Department of Chemistry, Biology, and Biotechnology, University of Perugia, 06123 Perugia, Italy
3 Istituto Officina dei Materiali, Italian National Research Council (IOM-CNR), Unit of Perugia
4James Watt School of Engineering, University of Glasgow, Glasgow G12 8LT, UK
alessandraanna.passeri@studenti.unipg.it

Spectroscopic techniques offer important advantages when it comes to the analysis of biological samples, particularly due to their contact-free and label-free nature, allowing extraction of sample information without causing damage. Here we present the combined Raman and Brillouin acquisition system developed in our Lab [1]. Raman Spectroscopy is a well-established technique able to extract the chemical composition of the sample by investigating the molecular vibrations. On the other hand, Brillouin spectroscopy is a technique able to determine the mechanical properties by measuring the spontaneous acoustic waves present in the material. Traditionally, Brillouin spectroscopy was applied in condensed matter physics to investigate homogeneous samples. However, in recent years, technological advances allowed to couple confocal microscopes to Brillouin spectrometers, achieving higher spatial resolutions. Enabling the mechanical characterization of single living cells and tissues, BLS is becoming an emerging technique in mechanobiology [2].
However, extracting the elastic properties from Brillouin data is not straightforward, because an accurate determination of refractive index (n) and density (ρ) is required in advance to relate the elastic modulus (M') to the Brillouin frequency shift ω ($M'=\rho\omega^2\lambda^2/4n^2$). These properties can vary due to sample fixation process [3]. Fixation is commonly employed before Brillouin analysis to stabilize samples and facilitate the slow-paced Brillouin spectroscopy measurements. Interestingly, we have discovered a variation of Brillouin shifts between fixed and fresh cells, raising questions regarding the potential impact of fixation on both frequency shift and mechanical properties. Therefore, employing a multi-technique approach, we investigated whether the modifications in refractive index and density alone drives the observed changes in frequency shift or if fixation induces alterations in mechanical properties as well.

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LIPID NANOPARTICULATE SYSTEMS CHARACTERIZATION THROUGH X-RAY SCATTERING TECHNIQUES

Alessia Pepe[1], Walter Pula[2], Agnese Bondi[2], Paolo Mariani[1], Maria Grazia Ortore[1], Elisabetta Esposito[2]

[1] Department of Life and Environmental Sciences - Marche Polytechnic University, Ancona, Italy;
[2] Department of Chemical, Pharmaceutical and Agricultural Sciences, University of Ferrara, Ferrara, Italy.

Lipid-based nanosystems (LBN) represent an attractive strategy for delivery of active ingredients characterized by low bioavailability, whose therapeutic potential might not otherwise be exploited. Indeed, LBN are characterized by high biocompatibility, ease of production, capability to encapsulate lipophilic molecules and ability to control their release.

Certainly, in this research area the characterization of these nanosystems is essential to ensure quality, safety and to enable the rational development of nanomedicines. In this regard, the objective of this work is to highlight the key role of X-Ray scattering technique in the determination of the internal structure of the lipid-based formulations, as well as the spatial distribution and phase behaviour of the supramolecular components.

Specifically, small and wide angle X ray scattering (SAXS and WAXS) allowed us to recognize the lamellar inner organization of solid lipid nanoparticles and of nanostructured lipid carriers, the multilamellar structure of ethosomes and to characterize lipid- and poloxamer-based gels [1-4]. In this case, rheological and X-ray scattering measurements evidenced the different thermoresponsive behaviors and supramolecular organizations of the semisolid forms [4]. Beside, SAXS was also essential to distinguish the different liquid-crystalline phases in which monoolein organized by the addition of different amounts of water (5-25% w/w) for the delivery of crocetin [5].

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HSP70 INHIBITORS AS THERAPEUTIC TARGET FOR CLCS GENETIC DISEASES

Michele Fiore,[1] Laura Lagostena,[1] and Alessandra Picollo[1,2]

1 IBF,Istituto di Biofisica - CNR, via de Marini, Genova, Italy
2 RAISE Ecosystem, Genova, Italy
alessandra.picollo@ibf.cnr.it

Defects in the proteostasis network that control protein production, folding trafficking, and recycling can cause genetic diseases. We focus our research on the CLC chloride protein family. CLCs, expressed in several tissues, are implicated in important physiological processes including skeletal muscle excitability, transepithelial salt reabsorption, cell volume regulation, and acidification of intracellular compartments[1]. Defects in CLCN genes cause muscle (myotonia congenital), bone (osteopetrosis), kidney (Barter syndrome, Dent's disease) and neurodegenerative disorders. CLCs missense mutations impairing the sorting of individual proteins or inactivating the protein trafficking machinery have severe pathophysiological consequences. It has been shown that by modulating the activity of heat shock proteins responsible for folding and assembling of CFTR, it is possible to restore the normal level of CFTR mutants in the plasma membrane[2]. Using a combination of electrophysiological, biochemical, and fluorescence assays, we will focus our study on two CLC proteins: the chloride renal channel ClC-K, and the lysosomal exchanger ClC-7. To explore the potentiality of using heat shock inhibitors as molecular targets for the recovery of protein cellular functionality of mutated CLCs, we selected two new Barttin mutants causing severe Bartter type IV[3], and two ClC-7 mutations causing osteopetrosis[4,5]. We determined which cellular and transport alterations affect protein functionality, and then we tested several Hsp70 inhibitors. We identified a molecule that was able to partially restore the proper protein functionality. The final goal is to identify new pharmacological targets for the treatment of genetic diseases caused by defects in CLCN genes and provide tools for functional classification of disease-causing mutants.

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RAMAN SPECTROSCOPY STUDY OF X-RAYS IRRADIATED NEUROBLASTOMA (SH-SY5Y) CELLS

Valentina Pisano,[1,2] Roberta Meschini,[2] and Ines Delfino[1]

[1] Dipartimento di Scienze Ecologiche e Biologiche, Università degli Studi della Tuscia, I-01100 Viterbo, Italy;
[2] Dipartimento di Economia, Ingegneria, Società e Impresa, Università degli Studi della Tuscia, I-01100 Viterbo, Italy;

Ionizing radiation is widely used in the medical field, especially in the treatment of tumors (such as radiotherapy) and in the diagnosis of various diseases. Despite the benefits of ionizing radiation in detecting and treating diseases, its use has significant potential adverse effects on living cells or tissue. These effects depend on several factors such as the type of radiation (such as X-rays, gamma rays, protons) and its characteristics, the type of tissue or cell receiving the radiation, and the radiation dose.[1] Several studies are available on DNA damage induced by ionizing radiation, but little is known about the effect of this radiation on other components, such as proteins and lipids. Recently vibrational micro-spectroscopy, including Raman microspectroscopy,[2,3] has been used to study irradiated cells. In this research, we report the preliminary results of Raman spectroscopy study of irradiated SH-SY5Y neuroblastoma cells, a human tumor cell line important in neuroprotection studies. Single fixed cells irradiated with graded doses of X-rays in the range of medical interest were investigated. The analysis of Raman spectra allowed us to detect subtle changes in the Raman fingerprints of proteins, lipids, and nucleic acids due to X-rays. The obtained complex spectra were analyzed in terms of Lorentzian line shape contributions to determine the optimized intensity, position, and linewidth of the peaks. Some peculiar Raman intensity ratios were then calculated. The results are discussed in terms of the possible translocation of C-cytochrome in the nucleus and the changes in protein structure and arrangements and membrane characteristics.

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NUTRANEURO – FUNCTIONAL EVALUATION OF NUTRACEUTICAL NEUROPROTECTION OF NEURONAL ION CHANNELS IN NEURODEGENERATIVE DISEASES: A PUTATIVE MOLECULAR TARGET FOR NEURORESILIENCE

Vera Plakhova[1], Ingrid Battistella[2], Marta Marchioretto[1], Linda Masello[3], Luciano Conti[2] and Carlo Musio[1]

[1]Institute of Biophysics (IBF), Trento Unit, National Research Council (CNR), Trento, Italy
[2]Department of Cellular, Computational and Integrative Biology—CIBIO, University of Trento, Trento, Italy
[3]Salix – Nutraceutical Thinking SRL, Monte di Malo, Vicenza, Italy
carlo.musio@cnr.it

Neurodegenerative diseases (NDDs) are irreversible and incurable disorders of the nervous system that cause progressive degeneration, up to the death of neurons. In NDDs, the analysis of neuronal excitability generated by ion channels is crucial for monitoring the disease. The pathogenic role of ion channels lies in the alteration of the intrinsic excitability of the cell and in the pathophysiological signs of disease. In previous works on SBMA - Spinobulbar Muscular Atrophy1-3 we have: 1) proposed the alteration of neuronal excitability, due to the dysregulation of ion channels, as a marker of the pathological phenotype of the disease; 2) demonstrated the role of various drugs in improving the symptomatic picture of the disease through the amelioration of altered excitability. Here we present preliminary data of the NutraNeuro Project, funded by Fondazione CARIVERONA (Verona, Italy), which combines the use of different cellular models (e.g., hIPSC stem cells also from patients, human NPC neurons, MN-1 pathological lines, etc.) of different NDDs (e.g. SBMA, SMA-Spinal Muscular Atrophy, ALS-Amyotrophic Lateral Sclerosis, etc.), and biophysical-pharmacological approaches. The project is aimed to establish in NDDs whether natural and/or nutraceutical compounds (e.g. curcumin, saffron, tea catechins, taurin, etc.) can exhibit neuroprotective effects, acting as symptomatic rescuers through the regulation of the ion channels' activity. Thus, applying patch-clamp, combined with pharmacology and cell neurobiology, to the above model cells, we are aimed to achieve: 1) the characterization of the alterations neuronal activity "fingerprints" underlying the membrane excitability; 2) the unveiling of the mechanisms of neuroprotection; 3) the evaluation of putative neuroresilient effect of nutraceuticals in single cells and/or cell clusters. Our combined approach will allow to obtain a) reliable preclinical models for the detection of neuronal excitability alteration, and its amelioration; b) eventual neuroprotective nutraceutical options in support to (neuro)therapeutic strategies. [Granted by Project "NutraNeuro", ReS 2022, Fondazione CARIVERONA]

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EARLY ELECTROPHYSIOLOGICAL STUDY OF THE EFFECTS OF CURCUMIN ON ION CHANNELS OF MOTONEURON-DERIVED CELLS MODELLING SBMA

Vera Plakhova[1], Vladimir A. Martínez-Rojas[2], Marta Marchioretto[1], Antonella Falconieri[3], Maria Pennuto[3] and Carlo Musio[1]

1 Institute of Biophysics (IBF), Trento Unit, National Research Council (CNR), Trento, Italy
2Pharmacobiology Department, CINVESTAV, Mexico City 14330, Mexico
3 Department of Biomedical Sciences (DSB), University of Padova, Padova, Italy
vplakhova@fbk.eu

Curcumin (CUR), a bioactive compound found in the rhizome of the turmeric plant (*Curcuma longa*), has gathered considerable attention in recent years due to its claimed health benefits, including anti-inflammatory, antioxidant, neuroprotective properties. From traditional medicine to modern research, CUR has been studied for its potential therapeutic properties being successful in preclinical trials for e.g. inflammatory, cardiovascular, skin, metabolic diseases, and in particular for neurological disorders[1]. The spinal and bulbar muscular atrophy (SBMA) is a rare X-linked recessive motor neuron disorder in adults caused by an expansion of CAG trinucleotide polyglutamine repeats of the androgen receptor (AR) gene, which results in loss of AR function causing motoneuron loss, muscle denervation, and skeletal muscle damage. The dysregulation of ion channels activity and the altered neuronal excitability in affected neurons has been identified as a key factor in the pathophysiology of this illness and a putative pharmacological target for therapeutic options[2-3]. Therefore, the aim of the current work was to investigate the possible nutraceutical effect of CUR on the ion channels underlying the neuronal functional activity of motoneuron-derived (MN-1) cells, which model SBMA. Whole-cell patch-clamp was performed on MN-1 cells stably transduced with lentiviral vectors expressing the human AR transgene that includes 24Q polyglutamine tract (AR24Q) (non-pathological) or the extended 100Q tract (AR100Q) (pathological), and treated before recordings with 10 μM or 20 μM CUR solution extracellularly applied. The data obtained showed that the application of CUR leads in AR100Q cells to a decrease of the macroscopic current, in particular of the outward voltage-gated currents mostly belonging to potassium. Though, a decreasing effect was observed on the sodium currents after 20 μM CUR application. Thus, in MN-1 polyQ AR cells we could conclude that CUR produces a reduction effect on altered neuronal excitability, inhibiting voltage-gated currents. Further investigations will confirm the role of CUR within neurotherapeutics based on ion channel-targeting pharmacology. [Granted by Project "NutraNeuro", Fondazione CARIVERONA]

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EFFECTS OF C282Y MUTATION IN HFE PROTEIN STRUCTURE AND INTERACTIONS, INVESTIGATED BY MD SIMULATIONS: IMPLICATION IN HEREDITARY HEMOCHROMATOSIS

Eugenia Polverini, Alice Trivelli and Elisabetta Ellettari

Department of Mathematical, Physical and Computer Science, Parco Area delle Scienze, 7/A, 43124, Parma, Italy
eugenia.polverini@unipr.it

Hereditary hemochromatosis (HH) is an autosomal recessive disease characterized by an improper regulation of iron uptake, leading to an iron overload in different specific organs. The responsible of HH is a mutated protein called HFE, formed by two domains: the α1,2 MHC-like domain and the α3 immunoglobulin-like domain.[1] Wilde type HFE competes with iron-loaded transferrin (Tf) for binding to the transferrin receptor (TfR) at the cell surface. HFE interferes with Tf binding site on TfR and regulates iron absorption. The mutated protein is not able to bind TfR and the regulation fails, causing the iron overload.[2] The predominant mutation in HH is the C282Y (C260Y in the mature protein) in the α3 domain, which converts a cysteine residue in a tyrosine, breaking a disulphide bond and affecting the association of HFE with a β2-microglobulin (β2m) chain, preventing its externalization at the cell surface and its binding with TfR.[3]

In this work, we investigated the effects of C282Y (C260Y in the mature protein) mutation on the HFE structure and on its interaction with the β2m domain, by means of molecular dynamics simulation technique. We followed the evolution of the HFE-β2m complex in the wt and mutated form, accelerating the structural changes by means of a temperature increment.

The mutated tyrosine comes to be in a hydrophobic environment and tries to move towards a more polar one enlarging the barrel, helped in this by the broken disulphide bond. PCA analysis allows to investigate how the reciprocal interactions of α3, β2m and MHC domains are affected by these conformational changes. Many conserved contacts are lost, but the main effect is the exposition of Trp60 residue of β2m, known to be fundamental for the association of β2m with the HFE chain.

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A BIOTECHNOLOGICAL APPROACH FOR LOADING MACROMOLECULES INTO EXTRACELLULAR VESICLES

Estella Rao,[1] Angela Paterna,[1] Giorgia Adamo,[2] Sabrina Picciotto,[2] Samuele Raccosta,[1] Antonella Bongiovanni,[2] and Mauro Manno[1]

[1] National Research Council of Italy (CNR), Cell-Tech Hub and Institute of Biophysics, Via Ugo La Malfa 153, 90146 Palermo, Italy

[2] National Research Council of Italy (CNR), Cell-Tech Hub and Institute for Research and Biomedical Innovation, Via Ugo La Malfa 153, 90146 Palermo, Italy

estella.rao@ibf.cnr.it

Extracellular vesicles (EVs) are biogenic nanoparticles originated from different cells to mediate cell-to-cell communication. Due to their intrinsic capability to vehicle biological materials and information, they have a high potential as drug delivery systems. We aim to set up a biotechnological platform for lab-scale vesicle production and exogenous loading of relevant molecules. Here we focus on the loading of large-size macromolecules, namely proteins and ds-dna (plasmids), which is still challenging in comparison to the encapsulation of small drugs. We use EVs derived from both mammalian cell (such as HEK-293T) and microalgae (named nanoalgosomes and derived from the microalgae *Tetraselmis chuii*) and different model molecules, including the green fluorescent protein (GFP), that is particularly suitable due to its intrinsic fluorescence. Our process optimization includes the following steps: (i) EV isolation from cell cultures by using TFF, a gentle filtration techniques that allows a scale-out of EV production; (ii) methods to perturb EV membrane and induce molecule encapsulation; (iii) purification of loaded product by different chromatographic techniques; (iv) structural and physico-chemical characterization by using different biophysical techniques; (v) assessment of EVs biological activities in order to validate and control their function and their mode of action. The biophysical and biochemical characterization of loaded EVs allows to validate the biotechnological approach for efficient loading of large cargoes into extracellular vesicles, confirming that nanoalgosomes are biocompatible EVs suited to carry not only small cargoes but also large-size macromolecules.

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CHEMORESISTIVE GAS SENSORS FOR CANCER DETECTION

Giulia Zonta[1,2], Giorgio Rispoli[3], Cesare Malagù[1,2] and Michele Astolfi[1,2]

[1]Department of Physics and Earth Science, University of Ferrara, 44122 Ferrara , Italy;
[2]SCENT S.r.l. , 44124 Ferrara, Italy
[3]Department of Neurosciences and Rehabilitation, University of Ferrara, 44121 Ferrara , Italy
rsg@unife.it

A patented device, based on nanostructured chemoresistive gas sensors, was employed to explore the gaseous exhalations (VOCs) of tumoral, immortalized, and healthy cell lines, with the aim of distinguishing their VOC patterns. The analysis of the device output to the cell VOCs, emanated at different incubation times and initial plating concentrations, was performed to evaluate the device suitability to identify the cell types and to monitor their growth. The sensors ST25 (based on tin and titanium oxides), STN (based on tin, titanium, and niobium oxides), and TiTaV (based on titanium, tantalum and vanadium oxides) used here, gave progressively increasing responses upon the cell density increase and incubation time; the sensor W11 (based on tungsten oxide) gave instead unreliable responses to all cell lines. All sensors (except for W11) gave large and consistent responses to RKO and HEK293 cells, while they were less responsive to CHO, A549, and CACO-2 ones. These encouraging results led us to employ this device to detect the VOCs of a primary colorectal cancer (CRC) sample and of a healthy sample (both of same weight, collected during colorectal surgery from the intestine of the same patient). The principal component analysis on the sensor responses to samples removed from 13 patients, indicate that the nanostructured sensors employed in this study were able to distinguish between healthy and tumor tissue samples with coherent responses (the discrimination power of the most sensitive sensor resulted of about 17%), highlighting a strong potential for the clinical practice. An upgraded version of this device has been then employed to analyse exhalations of blood samples, collected from CRC affected patients, at the following stages of their pre- and post-surgery therapeutic paths: the same day of the surgical treatment (T1); before the hospital discharge (T2); after at least one month after surgery (T3), and after 10-12 months from the surgery (T4). This device, was able to distinguish between T1 and T4 with a sensitivity of 93% and a specificity of 82%. The ability to detect the VOC pattern changes during the above stages, will allow a future use of this device in the clinical follow-up protocols in order to monitor the patient health status, and to detect possible future relapses from the surgical treatment.

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THE VOLTAGE-DEPENDENT IONIC CHANNELS OF THE ZEBRAFISH RETINAL CONES RESEMBLES THE ONES OF VESTIBULAR HAIR CELLS

Martina Masin,[1] Alessandra Attolico ,[1] Michele Garbellini,[1] Elena Frigato,[2] Paolo Spaiardi,[3,4] Sergio Masetto,[3] and Giorgio Rispoli[1]

[1]Department of Neuroscience and Rehabilitation, University of Ferrara, Ferrara, Italy.
[2]Department of Life Sciences and Evolution, University of Ferrara, Ferrara, Italy.
[3]Department of Brain and Behavioural Sciences, University of Pavia, Pavia, Italy.
[4]Department of Biology and Biotechnology, University of Pavia, Pavia, Italy.
rsg@unife.it

Vertebrate visual phototransduction represents a powerful model system for investigating G-protein signalling, and to understand animal vision because it is ultimately limited by the photoreceptor performance[1]. Vertebrate vision is initiated by the light-induced, transient suppression of the cationic current flowing into the outer segment of the retinal photoreceptor through the cGMP-gated channels in its plasma membrane. This current causes the photoreceptor hyperpolarization, whose waveform is shaped by the voltage-gated channels entirely segregated in the cell inner segment. These channels have been studied in zebrafish cones for the first time by recording them in whole-cell voltage-clamp. These animals are an ideal model since they share 70% of their genome with the human one, their embryonic development is remarkably fast, and huge resources for their genetic engineering are available. The photoresponses from mechanically isolated cones[2] were similar to the ones obtained from cones still inserted in a retina piece, therefore the mechanical isolation did not altered them. As in Type I vestibular hair cells[3], a depolarization elicited an outward rectifying K^+ current with a solution containing just ions with the appropriate concentrations, which activated with a kinetics that was at least as fast as the depolarization. After a few minutes of recording, a second component of this current manifested, with a voltage-independent activation of 3 ms. This suggests that this current is modulated by intracellular factors: one possibility is that the absence of nucleotides in the intracellular solution caused the progressive turn-off of a K^+ channel modulatory pathway aimed to the decrease its conductance. Blocking the K^+ current with Cs^+ and TEA^+, led to the appearance of a Ca^{2+} current, with a current-voltage relationship (IV) similar to the one of type II vestibular hair cells[3,4], and a second one, with a linear IV inverting at 0 mV, possibly carried by Cl^-.

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EXPLOITING AFM AS A NANOLITOGRAFIC TOOL: DESIGNING DNA PATCHES FOR PARALLEL DETECTION OF OLIGONUCLEOTIDE SEQUENCES

S.M.C. Rotondi[1], P. Canepa[1], M. Canepa[1,2], O. Cavalleri[1]

[1] Department of Physics, University of Genova, Via Dodecaneso 33, 16146 Genova, Italy
[2] INFN, Sezione di Genova, Via Dodecaneso 33, 16146 Genova, Italy silviamariacristina.rotondi@edu.unige.it

We designed a sensing platform for detecting disease-related oligonucleotides by immobilizing thiolated DNA single strands (probe DNA) onto a flat gold surface. These strands are complementary to the target sequence of interest. We conducted a comprehensive characterization of the platform coupling spectroscopic ellipsometry (SE), quartz crystal microbalance with dissipation (QCM-D), X- ray photoemission Spectroscopy (XPS), and Atomic Force Microscopy (AFM)[1,2].
Parallel detection of different sequences is crucial for a reliable diagnosis in different pathologies, from virus-related infections to cancer screening. Toward multiplex sequence targeting, we employed AFM nanolithography to graft platforms of different DNA probes. By applying a high tip load while scanning onto a selected region, it is possible to replace a previously chemisorbed inert alkanethiol SAM with thiolated probe DNA. By tuning the scanning parameters, grafted DNA patches with different molecular densities can be obtained. AFM Quantitative Imaging (QI) mode, a low perturbative technique, enabled the observation of coexisting molecular domains of different density within the grafted DNA patches. Subsequently, patches were exposed to the target sequence. An increase in height was measured for all patches, proving the hybridization between complementary strands. Preliminary results showed a detectable height increase down to 1nM target solution. Comparison of patch heights before and after hybridization revealed a higher relative height increase in the less dense patches, suggesting their suitability for targeting oligonucleotide sequences[3]. This approach can be leveraged to graft different probe sequences on the same substrate, enabling parallel and multiplex detection of different target sequences. Experiments using different sequences characterized by ten mismatches demonstrated the selectivity of each platform.

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ENGINEERING A ROS-GATED K^+ CHANNEL FOR THE TREATMENT OF NEUROPATHIC PAIN

Alberto Russo[1], Andrea Saponaro[2], Simone Trini[1], Rebecca Oddone[3], Chiara Villa[3], Yvan Torrente[3,4], Tyler S. Nelson[5], Rajesh Khanna[5], Gerhard Thiel[6] and Anna Moroni[1]

[1]Department of Biosciences, University of Milan, Milan, Italy
[2]Department of Pharmacological and Biomolecular Sciences, University of Milan, Milan, Italy
[3]Stem Cell Laboratory, Dino Ferrari Center, Department of Pathophysiology and Transplantation, University of Milan, Milan, Italy
[4]Neurology Unit, Fondazione IRCCS Ca' Granda Ospedale Maggiore Policlinico, Milan, Italy
[5]University of Florida College of Medicine
[6]TU-Darmstadt, Germany

Neuropathic pain is associated with overproduction of ROS (1) and is characterized by abnormal neuronal excitability. We have previously shown that light-controlled opening of a synthetic K^+ channel, BLINK2, limits neuronal excitability and has a marked analgesic effect in rat nociceptors (2). In this study, we designed an innovative synthetic K^+ channel that opens in response to a supraphysiological amount of ROS. Starting from the leaky channel mTASK-1 that is naturally modulated by ROS (3,4), we removed by rational mutagenesis the leaky background activity, allowing the channel to be gated only by ROS and we called the new construct ROS-TASK. ROS- TASK characterization was carried out by patch clamp combined with mutational analysis leading to the identification of the two methionine residues in the X-gate responsible for the ROS response. *In-vivo* experiments showed that AAV- mediated intrathecal delivery of ROS-TASK to DRG, induces robust pain relief in a rat model of neuropathic pain without inducing any effect in control animals. Moreover, mitochondrial- targeted ROS-TASK decreases ROS levels in a cellular model of mitochondrial myopathy with an elevated baseline concentration of ROS. In conclusion, our data demonstrate that ROS-TASK opens at supraphysiological levels of ROS and represents a tool for medical conditions associated with oxidative stress in which the opening of a K^+ conductance is beneficial.

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MINFLUX NANOSCOPY: PRELIMINARY RESULTS AND PERSPECTIVES AT SEELIFE INFRASTRUCTURE

Marco Salerno,[1] Virginia Bazzurro,[1] Elena Angeli,[1] Elena Gatta,[1] Paolo Bianchini,[2] and Alberto Diaspro[1,2]

[1]Dipartimento di Fisica, Università di Genova, via Dodecaneso 33, 16146 Genova, Italy
[2]Istituto Italiano di Tecnologia, via Enrico Melen 83, 16152 Genova, Italy
marco.salerno@unige.it

At the Department of Physics of the University of Genova a commercial Minflux instrument by Abberior has been recently installed in the frame of the project SEELIFE.[1] This fluorescence microscopy technique is based on blinking fluorophores and localization approach, with expected 2 nm precision.[2] Such a performance can provide single molecule resolution in 3D with better accuracy than STED or STORM, yet with significant requisites of proper experiment design and sample preparation protocol. So far, only characterization of test samples such as nuclear pore complexes and - for two-colors imaging - mitochondria have been carried out, confirming the setup capabilities. Next we will move on to co-localization of FRET molecular systems involved in CLC operation.[3] In perspective, in one direction we will address chromatin in nuclei at different cell life stages and conditions (healthy vs pathological for selected diseases) to characterize the condensation intensity and its peak position with respect to the distance from the lamina.[4] Another direction of research will be the investigation of nanoparticle endocythosis, aiming to elucidating the specific mechanism depending on particle chemistry and size.[5] Thanks to the Minflux nanoscopy all these biological problems will be characterized by imaging with unprecedented spatial resolution, which will allow for deeper insight and fundamental understanding of the underlying principles.

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UNRAVELING THE ORIGIN AND EVOLUTION OF ONCOGENE-DRIVEN DNA DAMAGE: INSIGHTS FROM SINGLE-CELL IMAGING

Silvia Scalisi[1], Anna P. Privitera[1], Pier Giuseppe Pelicci[3,4], Mario Faretta[3], Gaetano Ivan Dellino[3,4], Alberto Diaspro[2,5], Luca Lanzanò [1,2]

[1]Department of Physics and Astronomy "Ettore Majorana", University of Catania, Catania, Italy
[2]Nanoscopy, CHT Erzelli, Istituto Italiano di Tecnologia, Genoa, Italy
[3]Department of Experimental Oncology, IEO, European Institute of Oncology IRCCS, Milan, Italy
[4]Department of Oncology and Hemato-Oncology, University of Milan, Milan, Italy
[5] DIFILAB, Department of Physics, University of Genoa, Genoa, Italy
silvia.scalisi@unict.it

Oncogene-induced DNA damage is a critical phenomenon driving the intricate landscape of tumorigenesis. The aberrant activation of oncogenes can unleash a cascade of molecular events that disrupt the finely tuned process of DNA replication, leading to the accumulation of stalled replication forks, DNA breaks, and the formation of abnormal DNA structures, such as R-loops. Consequently, cells constantly struggle to maintain genomic integrity by triggering the DNA damage response (DDR) pathway, which aims to detect and repair damaged DNA. However, the chronic activation of DDR in the presence of oncogenic stress can overwhelm the repair mechanisms, exacerbating genome instability and increasing the mutation rate. In this scenario, we exploit the U937-PR9 cell line, an in vitro model for acute promyelocytic leukemia that hosts a metal-inducible PML-RARα oncogene expression vector, to unravel the mechanisms of DNA damage formation induced by the oncoprotein. We combine multicolor confocal imaging with our custom-made analysis algorithms to characterize DNA damage foci quantitatively at the single-cell level. We measure several parameters, including the number of foci, their average size and average intensity. We also analyze the spatial distribution of foci with respect to proteins of interest or relevant cellular processes with a colocalization algorithm based on image cross-correlation spectroscopy (ICCS). Furthermore, we correlate DNA damage formation and distribution to the S-subphase of the cell cycle through 5-ethynyl-2'-deoxyuridine (EdU) imaging. Our approach allows us to identify discernible markers of DNA damage elicited by the oncogene, thus furnishing invaluable insights into the origin of oncogene-activated DNA damage. Moreover, we treat our cellular model with inhibitors targeting the poly(ADP-ribose) polymerase 1 (PARP1) to investigate the progression of oncogene-induced DNA damage in an altered DDR environment.

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AN HCN4 SPECIFIC NANOBODY FOR THE MODULATION OF PATHOGENIC CHANNELS

Atiyeh Sadat Sharifzadeh[1], Roberta Castelli[1*], Andrea Saponaro[2], Alessandro Porro[1], Luca Palloni[1], Pietro Mesirca[3], Gerhard Thiel[1,4], Anna Moroni[1]*

[1]University of Milan, Department of Biosciences, Italy
[2]University of Milan, Department of Pharmacological and Biomolecular Sciences, Italy
[3]Institut de Génomique Fonctionnelle, Université Montpellier, CNRS, INSERM, France
[4]TU-Darmstadt, Department of Biology, Germany
[*]These authors contributed equally

Hyperpolarization-activated cyclic-nucleotide gated (HCN) channels are the molecular determinants of the I_f/I_h current that is involved in the regulation of spontaneous electrical activity in both heart and brain. HCN dysfunctions have been linked to the insurgence of cardiac (HCN4) and neurological (HCN1, HCN2) disorders that require subtype specific treatments. By screening an available yeast display library of synthetic nanobodies[1] we have identified Nanobody 5 (NB5) which selectively binds to HCN4 with nanomolar affinity and facilitates pore opening by shifting the half-activation voltage ($V_{1/2}$) of ~9 mV to the right. The NB5 binding site, identified by mutational analysis in the S5-S6 extracellular loop, reveals the presence of a non-canonical coupling pathway between the voltage sensor and the pore domain in HCN channels. NB5 acts on native I_f in sinoatrial node myocytes, increasing action potential generation by ~30% . In HEK293 cells, NB5 fully restores the WT phenotype of a pathogenic HCN4 mutation found in a tachy-brady patient. NB5 seems a promising therapeutic tool as the first HCN4 specific drug for the modulation of dysfunctional HCN4 channels.

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UNVEILING PHENYLALANINE–MEMBRANE INTERACTION DYNAMICS: INSIGHTS FROM MOLECULAR DYNAMICS SIMULATIONS

Andrea Spitaleri,[1] Valeria Rondelli,[1] Diego Colombo,[1] Mario Salmona,[2] Paola Brocca[1]

[1]Department of Medical Biotechnologies and Translational Medicine - University of Milan, Via F.lli Cervi 93, 20054 Segrate (MI)

[2]Department of Molecular Biochemistry and Pharmacology, Istituto di Ricerche Farmacologiche Mario Negri IRCCS, Milano, Italy

andrea.spitaleri@unimi.it

Molecular dynamics (MD) simulations offer a powerful computational approach to explore molecular interactions with atomistic detail at cellular membrane interfaces, offering valuable insights into disease mechanisms. We aim to delve into the atomic level details of Phenylalanine (Phe) interaction with model membranes, simulating pathological conditions of the phenylketonuria (PKU) disease. PKU is an inherited metabolic disorder characterized by impaired Phe metabolism, resulting in neurological consequences associated to dismyelination in White Matter. The disease's molecular basis is believed to be connected to the accumulation of Phe, as aggregate or monomeric, in the blood and tissues, thus at the surface of cell membranes. By simulating double bilayer asymmetric model membranes composed of phospholipids and glycolipids, we explore Phe interactions at the extracellular membrane interface and its permeability across the membrane.

Specifically, we investigate how Phe interacts with monosialotetrahexosylganglioside (GM1) and galactosylceramide (GalCer) containing membranes, elucidating the role of sugars in modulating Phe interactions. By comparison with experimental data from SAXS, SANS, neutron reflectometry, NMR and calorimetry [1], we provide a comprehensive understanding of how Phe may interfere with membrane slipping during the normal wrapping process of myelin sheaths, offering insights into the pathophysiology of PKU disease at the stage of myelin formation in infancy.

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NANOINDENTATION AS A METHOD FOR DETECTING BIOMECHANICAL PROPERTIES OF 2D CELL MODELS AND HYDROGELS

Ana Svetić[1,2] *Francesco Piazza*[3] *Caterina Medeot*[2,4] *Sacco Pasquale*[3] *Serena Bonin*[4] *Pietro Parisse*[5] *and Loredana Casalis*[2]

[1]Department of Physics, University of Trieste, 34127, Trieste, Italy
[2]Elettra-Sincrotrone Trieste S.C.p.A, SS 14 Km 163.5 in Area Science Park, 34149, Basovizza, Trieste, Italy
[3]Department of Life Sciences, University of Trieste, 34127, Trieste, Italy
[4]Department of Medical Science, University of Trieste, 34149, Trieste, Italy
[5]Istituto Officina dei Materiali – CNR, SS 14 km 163.5 in Area Science Park, 34149, Basovizza, Trieste, Italy

Nanoindentation is a fundamental technique for probing the mechanical properties of soft matter *in situ*, encompassing materials such as hydrogels, cells or cell monolayers, and 3D models (i.e., spheroids, organoids, and microtissues) at the nanometer or submicrometric scale[1]. Utilizing an indenter tip, this method involves the controlled application of force onto the surface while simultaneously measuring the depth of penetration. This allows determination of various mechanical properties (hardness, elastic modulus, and viscoelastic behavior), as well as additional mapping of the measured mechanical properties across the investigated sample surface[2,3]. In this work, two different samples were investigated: 2D melanoma cell models and agarose hydrogels, with the aim to compare the relative stiffness of different structures, quantified by Young's elastic modulus. The measurements were conducted using a nanoindenter with an interferometric readout (Optics11 Life Chiaro). For 2D cell models, four different melanoma cell lines were observed (Colo38, A375, 18732, and Mugmel2). The objective was to discern any variability among these cell lines and to note any alterations in stiffness before and after delivery of 2C inhibitor-loaded lipid nanoparticles (LNPs). The second sample, agarose hydrogels, have emerged as an important regulator of cellular activities, modulating mechanical transmission and transduction processes. Ultimately, they may serve as substrates for culturing melanoma cells, with recent studies linking cell response to Young's elastic modulus[4]. Consequently, indentations were performed on three agarose samples (Gracilaria, Gelidium, and agarose for electrophoresis (EP)), characterized by different chemical composition and physical properties, to analyze elastic modulus variations based on agarose type, both before and after fibronectin (FN) coating. This study underscores the capacity of nanoindentation as a reliable method which may enrich the understanding of cell response to drug delivery, and mechanical heterogeneity of hydrogels – essential for their optimization for utilization on living cells and tissues.

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REVEALING THE COMPLEX STRUCTURE OF NG2 PROTEOGLYCAN THORUGH MULTI-COMPUTATIONAL APROACHES

Francesco Tavanti,[1,2] Giorgia Brancolini,[2] and Roberto Perris[1]

1Universita' di Parma, Parco Area delle Scienze 11/a, 43124, Parma, Italy
2 CNR NANO, Via Campi 213/A, 41125, Modena, Italy
francesco.tavanti@unipr.it

Gastric cancer is one of the highest contributor to global cancer incidence and mortality[1], inferring that clinical management of the advanced disease remains highly cumbersome. Immune checkpoint inhibition in various combinations with chemotherapy is progressively becoming a first-line approach for advanced and/or metastatic gastric cancer. This highlights a clear potential in pursuing the identification and functional validation of novel immunotherapeutic targets to be clinically exploited through the accompanying generation of more specific immunological agents. The proteoglycan NG2/CSPG4 (chondroitin sulfate proteoglycan 4) is a 2322 amino acids transmembrane protein with 3 extracellular domains (D1 to D3), a transmembrane and cytoplasmatic domains[2], whose enhanced expression in neoplastic gastric tissues has been suggested to be prognostic[3].
The main issue to design a specific antibody targeting NG2/CSPG4 resides on the knowledge of the protein secondary and tertiary structure, and on its movements under physiological conditions that determine the available regions for antibody binding. This information is poorly available from experimental studies due to: 1) the large size of NG2/CSPG4, 2) its unknown folding and interaction with the membrane and 3) the complete lack of computational works on this protein.
In order to elucidate the structural and kinetical features of NG2/CSPG4, we employed a multiscale approach based on computational approaches: from sequence modeling to atomistic and coarse-grained simulations and to advanced analysis tools. From the predicted models obtained from AlphaFold2[4], extensive molecular dynamics simulations showed a peculiar compact folding of the extracellular portion when anchored to the cellular membrane, leaving the D1 domain exposed to the possible binding with antibodies. Moreover, the Principal Component Analysis shows that movements are mainly localized on the exposed portion of the D1 domain and on the D3 domain in the correspondence of the hook folding, while domain D2 is less flexible due to its packing between D1 and D3. Our work will improve the targeting ability and specificity of antibodies in all cancer type where the overexpression of NG2 has been observed to strongly decrease the survival probability of patients[3].

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INVESTIGATION OF HETERODIMERIC CLC-3 / CLC-4 ENDOSOMAL CHLORIDE / PROTON ANTIPORTERS

Abraham Tettey-Matey[1], Elena Angeli[2], Margherita Festa[1], Francesca Sbrana[1], Elena Gatta[2], Michael Pusch[1]

[1]Istituto di Biofisica, CNR, Via De Marini 6, 16149 Genova, Italy
[2]DIFILab, Dipartimento di Fisica, Università degli Studi di Genova, Via Dodecaneso 33, 16146 Genova, Italy
michael.pusch@ibf.cnr.it

Endolysosomes are intracellular organelles with important roles such as protein homeostasis, clearance of extracellular material, and autophagy. They are characterized by an acidic luminal pH that is critical for proper function. Five members of the gene family of dimeric voltage-gated ChLoride Channels (CLC proteins) are localized to endolysosomes, carrying out anion/proton exchange activity[1]. Mutations in the genes encoding these vesicular CLCs (vCLCs) cause global developmental delay, intellectual disability, various psychiatric conditions, lysosomal storage diseases, and neurodegeneration, resulting in severe pathologies or even death[1-3]. Among the vCLCs, endosomal ClC-3, -4, and -5 share a high sequence homology, with ClC-3 and ClC-4 being most important in neurons[1]. While ClC-3 is stable in homodimeric form, ClC-4 requires ClC-3 to form stable heterodimers[4]. To test the impact of ClC-4 variants in ClC-3/ClC-4 heteromers, we co-injected in Xenopus oocytes WT ClC-3 RNA with WT ClC-4 RNA and the LoF function variants R360S, G545S, K560E, G731V, and G731R and measured transport currents by voltage-clamp[3]. R360S exerted an overall dominant effect, while G545S and K560E resulted in a voltage-dependent reduction of currents, indicating a dominant negative effect in heterodimers via a shift of the voltage-dependence of gating. Surprisingly, for G731V and G731R, which are LoF when injected alone[3], currents were increased in oocytes co-injected with ClC-3 and showed an altered voltage-dependence. Direct evidence for a physical interaction of ClC-3 with ClC-4 was obtained by Förster resonance energy transfer (FRET) measured by using fluorescence lifetime imaging microscopy (FLIM), the so-called FLIM-FRET method. The lifetime of GFP-tagged ClC-3 alone was ~2.4 ns. Upon co-transfection with mCherry-tagged ClC-4, the lifetime was reduced to ~2 ns, indicating close interaction of the two proteins. As negative controls, ClC-3-GFP lifetime was not reduced by ClC-1-mCherry, which is presumably unable to dimerize with ClC-3. Similarly, ClC-6-GFP lifetime was unaffected by co-expressed ClC-4-mCherry. FLIM-FRET analysis will be employed to test whether disease-associated ClC-4 or ClC-3 variants impact the dimerization process.

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EFFECT OF MECHANICAL STRESS AND HYDROPHOBIC INTERFACES ON PROTEIN NANOFIBRILS SELF-ASSEMBLY

Francesco Troisi[1], Davide Odino[1], Elena Angeli[1], Lorenzo Vannozzi[2], Sabrina Ciancia[2], Tommaso Mazzocchi[2], Leonardo Ricotti[2], Annalisa Relini[1], Claudio Canale[1]

[1]Department of Physics, University of Genova, Genova, Italy
[2]The BioRobotics Institute, Scuola Superiore Sant'Anna, Polo Sant'Anna Valdera, Viale Rinaldo Piaggio 34, 56025 Pontedera, Italy

Under suitable conditions, proteins can self-assemble in solution forming fibrillar structures with diameter of few nanometers and micrometer length. These nanofibrils, named amyloid fibrils, are associated with several pathological conditions, including Alzheimer's and Parkinson's diseases and systemic amyloidosis1. Understanding the molecular mechanisms of protein aggregation is crucial to develop appropriate therapeutic strategies. Several pieces of evidence suggest that the presence of hydrophobic interfaces and mechanical stress can indeed enhance protein aggregation2. Here we want to investigate the role of shear forces and hydrophobic interfaces in the aggregation process. We focus our attention on the aggregation process which takes place in cardiac tissue, that is a typical deposition site of amyloid fibrils associated with cardiac amyloidosis. A prototype of a device has been set up to mimic the cardiac environment in its chemical composition and mechanical behavior. Shear forces are applied to the protein solution under study either using a magnetic stirrer or exploiting contraction/expansion cycles of a flexible polymeric cell. This condition is coupled to exposure of the protein solution to hydrophobic interfaces. To this purpose, cell walls are functionalized with elastin, a typical protein present in the physiological environment of cardiac tissue which exposes hydrophobic region under stress. To set up the experimental conditions, k-casein3 was chosen as a protein model. Amyloid aggregation was studied both in the absence and in the presence of shear stress. Protein aggregates were characterized by atomic force microscopy (AFM) and fluorescence spectroscopy measurements. The experimental results obtained in the present work will yield insight into the mechanisms of protein aggregation under pathological conditions.

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DECIPHERING THE INTERACTIONS IN LIQUID-LIQUID PHASE SEPARATION: INSIGHTS FROM PROTEIN CONDENSATION

Rosa Bartucci[1], Rita Guzzi[1], Luca Lanzanò[2], Giuseppe Sancataldo[3], Valeria Vetri[3]

1 Dipartimento di Fisica - Università della Calabria
2 Dipartimento di Fisica e Astronomia- Ettore Majorana, Università degli Studi di Catania
3 Dipartimento di Fisica e Chimica- Emilio Segrè, Università degli Studi di Palermo
valeria.vetri@unipa.it

Biomolecular condensates are crucial for orchestrating cellular functions, being constituents of membranless organelles whose activity appears to be related to their fluid-like state. Nowadays, it is well assessed that Liquid-Liquid Phase Separation (LLPS) is the dominant phenomenon regulating the assembly and disassembly of multicomponent molecular condensates even if the understanding at molecular level of the assembly, dynamics and of the spatial organization of these structures is still lacking. In addition to the unique role of LLPS in cell function/dysfunction, the concept of emulating these processes for the design of new biomaterials tailored to various applications is now gaining traction. These applications include drug delivery, sensing, and the development of biomolecule gradients with responsive properties to stimuli and environmental factors.

The activities of the PRIN project "Liquid-Liquid Phase Separation dynamics in biomimetic compartments" (LLIPS) Project code: P20228CCLL are aimed at uncovering fundamental principles governing LLPS and, in particular, protein condensation in biologically relevant conditions. This will be achieved through optimizing a fully controllable synthetic proto-cell system, and employing a novel imaging/spectroscopic technique to observe structural organization and dynamics of biomolecules in compartments down to the nanoscale.

In this context, we here present the analysis of the LLPS of model proteins where solution conditions are regulated to stabilize protein condensate with different structural and stability features. The experimental approach is based on the combination of classical spectroscopy techniques and quantitative fluorescence microscopy which allow monitoring occurring events in real-time with high spatial resolution. Results highlight the role of electrostatic and entropic interactions in controlling protein condensates physico-chemical properties which can be regulated by solution conditions.

MOLECULAR INSIGHTS INTO NITRIC OXIDE SENSITIVITY OF FLUORESCENT PROTEINS

Carlotta Viappiani[1], *Lucia Bellanova*[1], *Pietro Delcanale*[1] *and Riccardo Nifosì*[2]

[1]Dipartimento di Scienze Matematiche, Fisiche e Informatiche, Università di Parma, Parco Area delle Scienze 7A, 43124 Parma, Italy.

[2]NEST, CNR-Istituto Nanoscienze and Scuola Normale Superiore, Piazza S. Silvestro 12, 56127 Pisa, Italy

carlotta.viappiani1@studenti.unipr.it, riccardo.nifosi@nano.cnr.it

Fluorescent protein (FP) variants have recently emerged as promising candidates for sensing nitric oxide (NO) intracellular concentrations [1]. These variants exhibit NO-induced loss of fluorescence, arising from cysteine S-nitrosylation, a process in which the NO molecule binds to the sulfur atom of the amino acid. Among these variants, mTagBFP2, a blue fluorescent protein derived from the orange FP eqFP578, demonstrates particularly strong sensitivity to NO, resulting in a 70% reduction of fluorescence quantum yield and lifetime. Here, we investigate possible mechanisms of fluorescence loss upon S-nitrosylation including perturbations in the protein structure, leading to increased chromophore flexibility and subsequent enhancement of non-radiative decay. Additionally, Forster resonant energy transfer (FRET) to the newly acquired NO group(s) is considered, as their absorption spectrum, characterized by $n \rightarrow \pi^*$ and $\pi \rightarrow \pi^*$ bands peaking at 545 and 335 nm respectively, partially overlaps with the emission spectrum of mTagBFP2. Through molecular dynamics simulations, we address the structural alterations upon NO binding and calculate the geometric factors necessary to accurately estimate FRET efficiency. Furthermore, we discuss how our theoretical and computational findings align with experimental measurements on mTagBFP2 and other FP variants.

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INCREASED GENETIC NOISE AND SHANNON ENTROPY DUE TO MONOALLELIC GENE EXPRESSION IN DEVELOPING CELLS

Robert Wolff,[1] Edoardo Balzani,[1,2] Alessia Polito,[1,3] Angelo Serani,[1] and Valter Tucci[1]

1Genetics and Epigenetics of Behavior Laboratory, Fondazione Istituto Italiano di Tecnologia (IIT), 16163 Genova, Italy
2Present address: Center for Neural Science, New York University, 10012 New York, NY, USA
3Padova Neuroscience Center (PNC), University of Padova, 35129 Padova, Italy
robert.wolff@iit.it

Monoallelic expression is a pivotal phenomenon in developmental biology, notably through the influence of imprinted genes on various processes, including cellular differentiation and brain development[1]. Our predictive model suggests that monoallelic expression generates genetic noise,[2] offering insights into brain cell organisation. Analysing single-cell allelic expression across diverse datasets[3], we consistently find increased genetic noise due to monoallelic expression. Notably, this effect extends beyond imprinted genes, affecting co-expressed non-imprinted genes, highlighting the pervasive impact of genomic imprinting throughout the genome. We found that this effect is context-dependent and unaffected by the parental origin of the allele. Moreover, we observed increasing genetic noise in the development of neurons while glial cells exhibited decreasing genetic noise. The discovery of distinct noise patterns in over 80% of analysed genes between glial and neuronal populations underscores the importance of differential noise in fundamental developmental processes. Then, we investigated gene expression variation within the framework of Shannon's information theory[4]. We found similar relations of increased Shannon entropy at monoallelic expression for both imprinted genes and their co-expressed non-imprinted genes. Since imprinted genes play crucial roles in regulating biological processes such as growth and brain development, disruptions in their expression can contribute to various disorders. Thus, unravelling the stochastic nature of monoallelic gene expression and its genome-wide implications holds promise for gaining new insights into the mechanisms underlying these pathologies.

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TRANSMEMBRANE CHANNEL-LIKE PROTEIN 1 (TMC1) IN PHYSIOLOGICAL AND DEAFNESS-ASSOCIATED STATES: INSIGHTS FROM MOLECULAR DYNAMICS AND FLUORESCENCE MICROSCOPY

Davide Zamboni[1], *Giuditta dal Cortivo*[1], *Valerio Marino*[1], *Gianluca Lattanzi*[2, 3], *Daniele Dell'Orco*[1]

1 Department of Neurosciences, Biomedicine and Movement Sciences, Section of Biological Chemistry, University of Verona, 37134 Verona, Italy.
2 Department of Physics, University of Trento, Trento, Italy
3 Trento Institute for Fundamental Physics and Applications, Istituto Nazionale di Fisica Nucleare, Trento, Italy
davide.zamboni@univr.it

Despite the lack of high-resolution experimental evidence, in recent years a general consensus on the function and structure of Transmembrane channel-like protein 1 (TMC1), the long-sought mechanotransductive protein channel initiating hearing in mammals, has been reached[1]. Recent lines of evidence point to a dimeric complex with one channel per monomer facing the membrane. We present a thorough characterization based on 1 μs Molecular Dynamics (MD) simulations of both wild-type and M654V-TMC1, a missense variant associated with non syndromic deafness[2]. The comparative analysis concerned e pore structures, ion distribution around the pore and the dipole momentum of each monomer, unveiling possible structural determinants responsible for the emergence of the photogenic phenotype. By means of the program HOLE[3], the geometrical features of the holes through the ion channel were assessed, providing information on the pore profile in terms of lipidic contribution to walls, electrostatic environment and pore size. The analysis highlighted different involvement of the membrane in pore formation, choke point and fluctuations of the pore centers for the two variants. Density maps of chloride and potassium ions around the protein revealed an altered ionic distribution in M654V-TMC, an aspect further confirmed by the analysis of the position of the slow-diffusing atoms in the two variants and essentially in line with the differences in the dipole momenta of the monomers. Finally, we present preliminary *in cellula* data on the putative interaction between TMC1 variants and its interactor Calcium- and Integrin-binding protein 2 by means of homologous expression in HEK293 cells and immunocytofluorescence analysis[4].

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LIPID LOWERING AND ANTIOXIDANT ACTIVITIES OF A POOL OF NATURAL BIOACTIVE COMPOUNDS: THE ROLE OF PPARS INVESTIGATED BY EXPRESSION AND MOLECULAR DOCKING ANALYSIS

Hawraa Zbeeb,[1] Francesca Baldini,[2] Farah Diab,[1] Velia Micozzi,[3] Lama Zeaiter,[1,2] Piero Portincasa,[5] Alberto Diaspro,[2,3] and Laura Vergani [1]

[1] DISTAV, Department for the Earth, Environment and Life Sciences, University of Genoa, Genoa, Italy
[2] Nanoscopy, Istituto Italiano Tecnologia, Genoa, Italy
[3] Department of Physics and INFN, Tor Vergata University of Rome, Rome, Italy
[4]DIFILAB Department of Physics, University of Genoa, Genoa, Italy
[5] Clinica Medica "A. Murri", Department of Biomedical Sciences and Human Oncology, University of Bari, Bari, Italy

The metabolic syndrome (MS) is a cluster of metabolic derangements that are associated with disturbances in adipose tissue leading to visceral obesity[1]. Existing drugs for treatment of MS and obesity possess only modest efficacy. Lifestyle modification is still the most effective strategy, but the lack of compliance is hampering this approach. Therefore, drugs and/or nutraceuticals with good efficacy and safe are urgently needed. Here, we focused on five natural compounds, three plant phenolic compounds (carvacrol, rosmarinic acid, and silybin), and two hormones (the thyroid hormones T2 and T3) to test and compare their beneficial effects on two cellular models of hepatosteatosis and adipose tissue. Their ability of counteracting the excess intracellular lipid accumulation and the fat-dependent oxidative stress in the two cell models was compared, in the attempt to clarify the distinctive molecular mechanisms underlying their beneficial action. The results indicate that all the bioactive compounds were able to significantly reduce the triglyceride accumulation and to improve the oxidative stress in both hepatocytes and mature adipocytes, but with different efficacy. For this reason, their binding to the peroxisome proliferator-activated receptors (PPARs) was tested by molecular docking calculations. PPARs are nuclear receptors controlling the expression of genes involved in lipid and carbohydrate metabolism. The results show that the five compounds bind the main isoforms PPAR-γ and PPAR-α with different affinity. In conclusion, the present results reveal some interesting insights into the mechanisms by which the five compounds may act as nutraceuticals, which could find application in strategies to prevent/counter obesity.

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