LABORATORY ACTIVITIES

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(Part A and Part B)

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(Part A and Part B)

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Microscopy

Activity 5

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Activity 6

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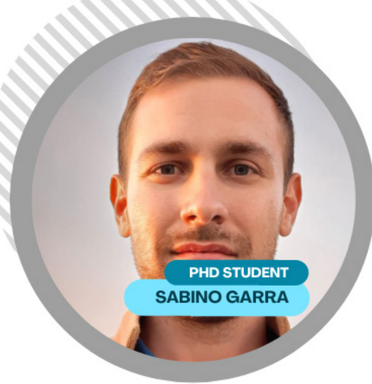
ACTIVITY 1 - PART A

TOTAL INTERNAL REFLECTION MICROSCOPY AND FLUORESCENCE-QUENCHING ASSAY FOR WATER TRANSPORT MEASUREMENTS ACROSS ASTROCYTE PLASMA MEMBRANE



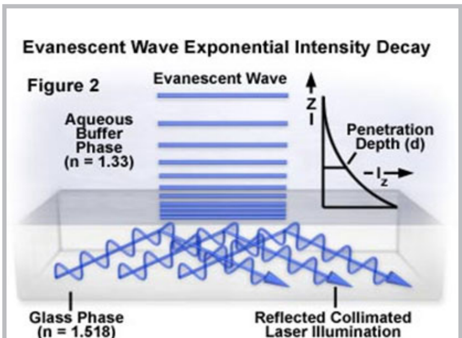
ACTIVITY 1 - PART B

STOPPED-FLOW LIGHT SCATTERING FOR MEMBRANE OSMOTIC PERMEABILITY MEASUREMENTS

Activity 1

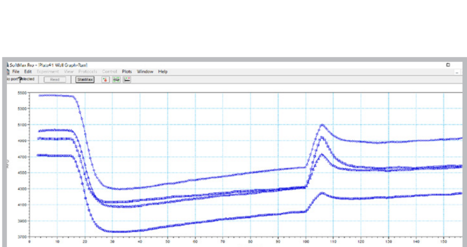
Water Transport Assays (Part A and Part B)

The rate of many biological processes often depends on the speed at which the molecules enter or leave the cell by crossing the plasma membrane. The measurement of the cell membrane permeability represents, in fact, an essential prerogative in the study of cellular functions under normal and pathological conditions. In the technical sessions, three of the gold-standard biophysical techniques routinely used to measure water membrane permeability of living cells, sealed membrane vesicles or liposomes will be introduced.

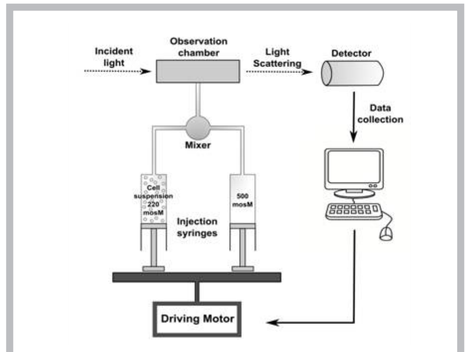


Part A) Total Internal Reflection Microscopy and Fluorescence-Quenching Assay for water transport measurements across astrocyte plasma membrane

Total Internal Reflection Microscopy (TIRFM) is a well-established method to study water and solute permeability in cells of arbitrary shape and size. Adherent cells labeled with an aqueous-phase dye are rapidly exposed to an osmotic gradient and the time course of fluorescence signal is detected. TIRFM uses an evanescent wave instead of direct illumination to selectively excite fluorophores in a very thin layer of cytosol adjacent to the glass-water interface. This allows the observation of membrane-associated processes and reduces dye photobleaching. This activity will allow the participants to use properly TIRFM experimental set-up that includes a conventional microscope equipped with a high-numerical-aperture objective, a laser source, and a cell perfusion system.



The Calcein based Fluorescence quenching assay provides another approach to evaluate water membrane permeability by measuring the concentration of an aqueous-phase fluorophore in cytoplasm. Cells are loaded with high concentrations of membrane-impermeant calcein dye to produce volume-dependent fluorescence signal in response to an osmotic challenge. Cytosolic calcein fluorophore exhibits concentration-dependent quenching by intra-cellular components (proteins or salts) so that measured changes in fluorescence are directly proportional to changes in cell volume. Calcein fluorescence intensity increases upon addition of hypotonic media due to water influx and cell swelling and decreases in response to hypertonic stimulation due to water efflux and cell shrinkage. In the training session, the fluorescence kinetics in response to altered extracellular osmolarity will be recorded on a benchtop fluorescence plate reader (FlexStation3, Molecular Devices) equipped with a liquid handling module able to transfer reagents from a source plate to the read plate during data acquisition. The participants will be introduced to the practical aspects of a medium throughput screening for potential modulators of water channel function in calcein labelled cells.



Part B) Stopped-Flow Light Scattering for membrane osmotic permeability measurements

The stopped-flow light scattering (SFLS) represents a sensitive, versatile, and reliable technique which uses a non-fluorescent approach to measure the permeability of biological and artificial membranes. In the training session, the SFLS set-up will be used to calculate the biophysical parameters of plasma membrane water transport as follows: a hyper- or hypotonic medium and a suspension of whole cells, artificial vesicles, or liposomes, both contained in separate drive syringes, are fired together into a mixing chamber. The mixed reactants pass an observation cell that allows the reaction to be followed by light-scattering. The variation in the scattered light intensity reflects the changes in volume of suspended particles (cells, vesicles, liposomes or polymersomes) induced by the osmotic shock. Therefore, computing the time constant (ki) of the kinetics in specific biophysical formulas, it is possible to trace the osmotic permeability coefficient to water (Pf) or to anaelectrolyte solutes (Ps).

Under the supervision of Prof. Grazia Paola Nicchia and Prof. Giuseppe Calamita, these technical activities will be held by Dr Maria Grazia Mola and Dr Guido Mogni (Part A) and by Dr Patrizia Gena and Sabino Garra (Part B)

ACTIVITY 2- PART A

ELECTROPHYSIOLOGICAL ACTIVITY OF ISOLATED CELLS: PATCH-CLAMP RECORDING FROM HL-1 CARDIOMYOCYTES

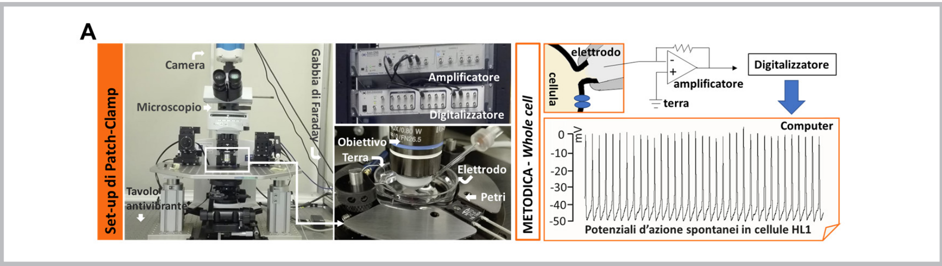
ACTIVITY 2 - PART B

ELECTROPHYSIOLOGICAL ACTIVITY OF POLARIZED EPITHELIA: THE USSING CHAMBER SYSTEM FOR MEASURING TRANSEPITHELIAL ELECTRICAL PARAMETERS

Activity 2

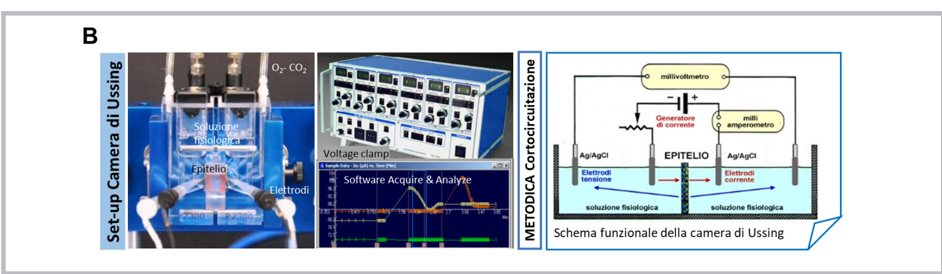
Electrophysiology (Part A and Part B)

Ion channels activity forms the basis of many physiological processes. Measuring ion currents across membranes of different experimental models allowed a clear understanding of the role of ion channels in a wide spectrum of cellular pathways. Different technical approaches have been developed throughout the years to measure the electrical properties of isolated cells and/or intact tissues or cell monolayers. Here, the electrophysiological laboratory experience and training will have a double aim; on one hand, to explore cardiac biophysical properties in single Hl-1 cardiomyocytes. On the other hand, to investigate the vectorial ion transports in a polarized monolayer of epithelial cells.



Part A) Electrophysiological activity of isolated cells: Patch-Clamp recording from Hl-1 cardiomyocytes

The students will be introduced to the proper use of our experimental set-up for patch clamping, assembled by Crisel Instruments, to study action potentials and ionic data from current-clamp and voltage-clamp recordings. In particular, the students will learn best practices for culturing cardiomyocytes, intracellular and extracellular saline solutions preparation, identifying cardiomyocytes for patch clamp, filling the recording pipette, forming the GΩ seal and recording action potentials and inward/outward currents.



Part B) Electrophysiological activity of polarized epithelia: the Ussing Chamber system for measuring transepithelial electrical parameters

Epithelia are polarized structures with an apical and a basolateral side. It is the movement of electrolytes, non-electrolytes, and water across epithelia that functionally characterize a tissue. Ussing systems have been used from decades to gain information from native tissue including stomach, intestine, bladder, skin, and trachea, as well as from tissue derived cell monolayers from various origins. The students will be able to learn the physiological principles that underlie the technique and protocols to investigate transepithelial biophysical parameters such as the short circuit current (the current required to cancel the potential difference of the tissue) and the resistance of cells to the passage of an ion flow.

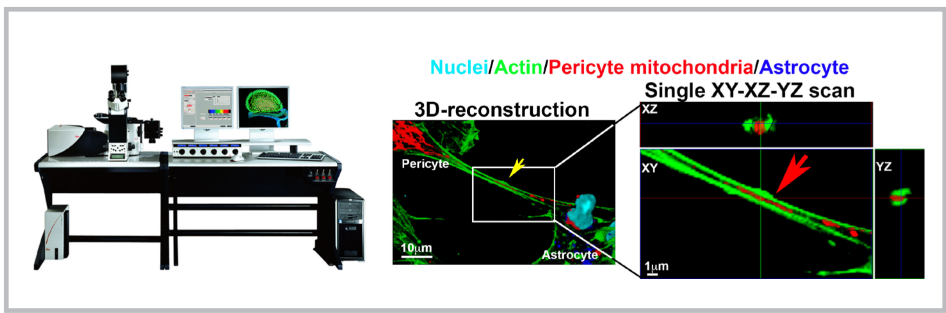
Under the supervision of Dr Andrea Gerbino, these technical activities will be held by Dr. Roberta De Zio and Dr. Fatima Maqud (Part A) and by Dr. Rosa Caroppo and Dr. Francesca Piccapane (Part B).

ACTIVITY 3

MULTICOLOR CONFOCAL LASER SCANNING MICROSCOPY AND IMAGE ANALYSIS OF HUMAN CO-CULTURES MODELS.

Activity 3

Confocal Microscopy



Multicolor Confocal Laser Scanning Microscopy and Image Analysis of human co-cultures models.

Co-culture models replicate the complexity of tissues and give the opportunity to investigate cell-to-cell crosstalk mechanisms in physiological and pathological conditions.
Aim of this laboratory experience and training will be the analysis of cell-to-cell crosstalk mechanisms in human blood-brain barrier coculture models by using Laser Scan Confocal Microscopy (LSCM) and image analysis software.
The participants will be introduced to the correct design of multicolor LSCM experiment using Leica TCS SP5 microscope. Technical issues and relative solutions will be presented to introduce the correct sample preparation, fluorophore combination, fluorescence preservation, acquisition of XYZ-series and 3D-reconstruction. Leica Application Suite X (LASX) and FIJI software will be presented and used at this scope.

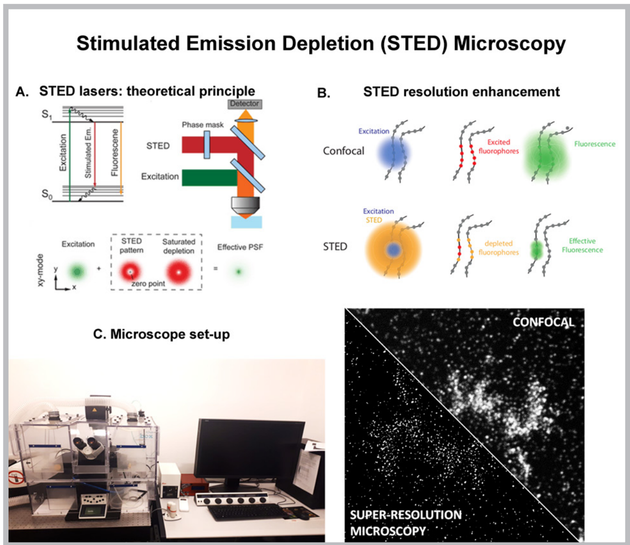
Under the supervision of Prof. Giuseppe Procino and Prof. Francesco Pisani, this technical experience will be held by Dr. Serena Milano and Dr. Ilenia Saponara.

ACTIVITY 4

STIMULATED EMISSION DEPLETION SUPER-RESOLUTION MICROSCOPY FOR THE ANALYSIS OF CHANNEL AGGREGATION INTO THE PLASMA MEMBRANE OF CELLS, TISSUES AND CENTRAL NERVOUS SYSTEM-LIKE BIOMIMETIC LIPID BILAYERS

Activity 4

Super-Resolution Microscopy



STimulated Emission Depletion super-resolution microscopy for the analysis of channel aggregation into the plasma membrane of cells, tissues and central nervous system-like biomimetic lipid bilayers

STimulated Emission Depletion (STED) microscopy is a fluorescence technique that overcomes the diffraction limited resolution of confocal microscopes. It consists of an advanced microscope design that uses a doughnut-shaped laser to sharpen the point-spread function (PSF) of the microscope and enhance confocal spatial resolution by an order of magnitude. In the practical training, participants will learn the theoretical basis of STED microscopy and its applications, become familiar with the components of the Leica TCS SP8 STED microscope and apply different configurations to image fixed samples and reach a lateral resolution below 50 nm for membrane protein aggregation analysis. Finally, participants will use Huygens software to perform dimensional analysis on collected data. All participants are welcome to bring and image their own fixed samples (for reference: https://www.leica-microsystems.com/science-lab/the-guide-to-sted-sample-preparation/).

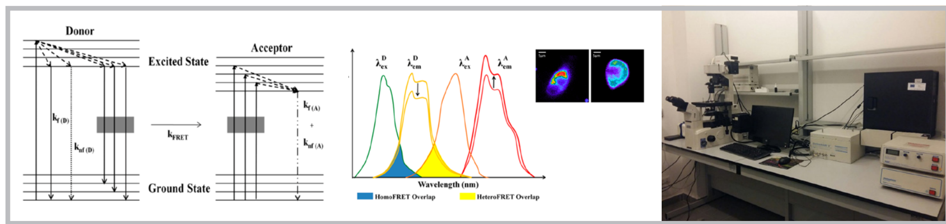
Under the supervision of Prof. Frigeri, this technical experience will be held by Dr Barbara Barile and Dr Antonio Cibelli.

ACTIVITY 5

DETECTING CAMP WITH FRET-BASED SENSORS IN SINGLE LIVING CELLS

Activity 5

Fluorescence Resonance Energy Transfer Microscopy



Detecting cAMP with FRET-based sensors in single living cells

Förster (or Fluorescence) Resonance Energy transfer (FRET)* is a distance-dependent physical transmission of energy from an excited fluorescent donor molecule to an acceptor molecule to which the energy is transferred through long-range dipole-dipole interactions. Based on its sensitivity to distance, FRET microscopy is used to observe molecular interactions, with high spatial and temporal resolution. The experience and training will consist in the real-time evaluation of cAMP changes in human embryonic kidney (HEK-293) living cells. The cAMP increase will be monitored in real time, under control condition and after stimulation with forskolin (FK). We will focus on the different compartmentalized measurements of cAMP in the cytoplasm, in the plasma membrane or in the intracellular organelles, using different Epac-based probes. The students will be instructed to the proper use of this set up assembled by Crisel Instruments around a Nikon inverted microscope, and consisting of a mercury lamp, a cooled enhanced ECCD camera and a computer with the software for the acquisition and analysis.

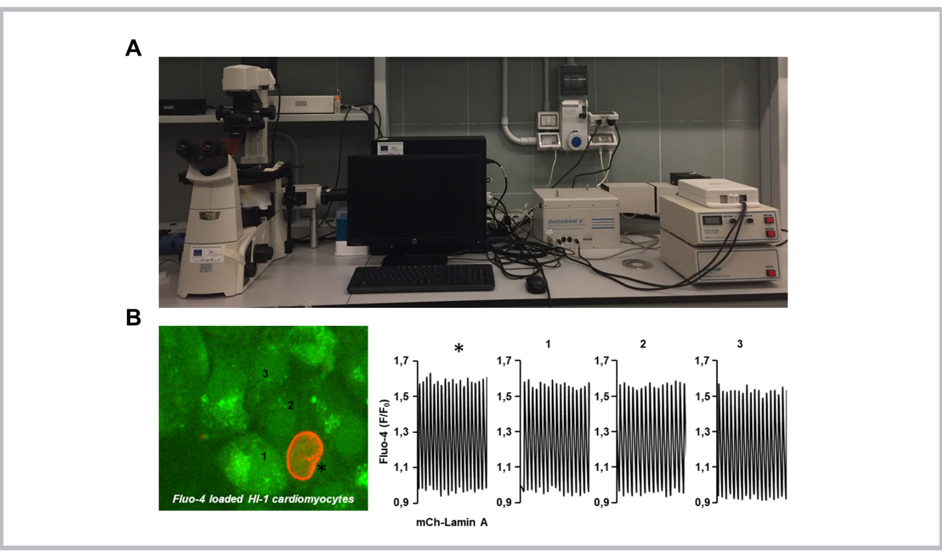
Under the supervision of Prof. Grazia Tamma, this technical experience will be held by Dr. Marianna Ranieri and Dr. Annarita Di Mise.

ACTIVITY 6

REAL TIME IMAGING OF CALCIUM SIGNALS IN SINGLE CARDIAC CELLS: A GOLD STANDARD TOOL IN CELL PHYSIOLOGY

Activity 6

Calcium Imaging



Real time imaging of calcium signals in single cardiac cells: a gold standard tool in cell physiology

The aim of this laboratory experience and training will be the real-time evaluation of cytosolic Ca2+ oscillations in cardiac cell models (such as HL-1 cells) loaded with Ca2+-sensitive dyes. The Ca2+ oscillation patterns will be monitored under control condition and after stimulation with physiological challenges that mimic either parasympathetic or sympathetic activation.
The student will be introduced to the proper use of our experimental set-up, assembled by Crisel Instruments, which is composed of a monochromator, a Nikon inverted microscope, a cooled CCD camera and a computer with the acquisition software. How to choose the fluorescent dye that best fit the researcher's project will be considered.

Under the supervision of Prof. Matilde Colella this technical experience will be held by Dr. Roberto Barbaro and Dr. Simona Scorza.