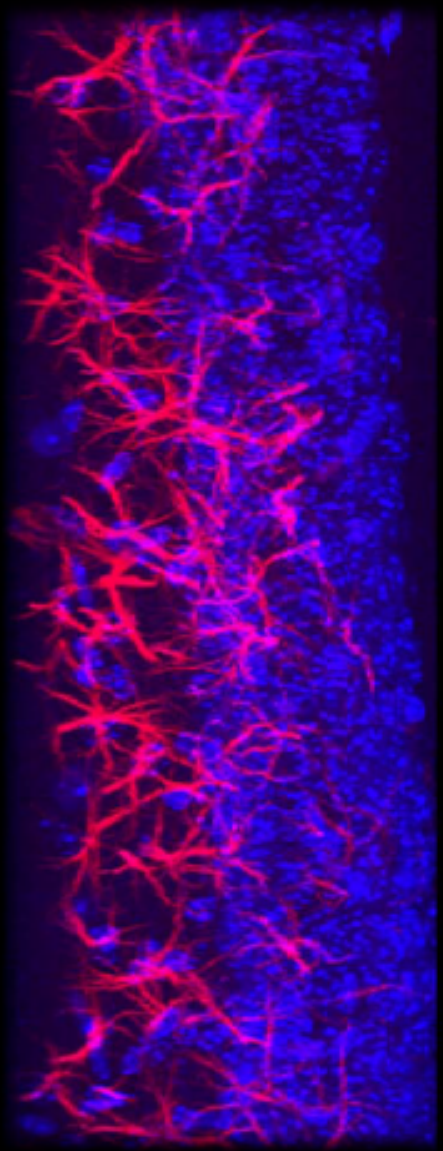
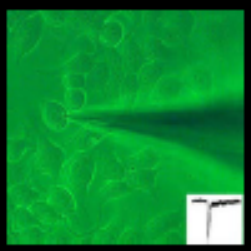
LIVE CELL IMAGING AND ELECTROPHYSIOLOGY



A Theoretical and Practical Workshop
Hellenic Pasteur Institute, Athens, Greece
1-4 October 2013



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DIMITRIOS DAVALOS, University of California
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FRANK KIRCHHOFF, University of Saarland
REBECCA MATSAS, Hellenic Pasteur Institute
CORNELIA POULOPOULOU, University of Athens
DAVIDE RAGOZZINO, University of Rome
PAVLOS RIGAS, BRFAA Athens
IRINI SKALIORA, BRFAA Athens
DIMITRA THOMAIDOU, Hellenic Pasteur Institute

A limited number of fellowships is available for students and young scientists

FP7 REGPOT 2010-1 Neurosign 264083

Organized by:
Dimitra Thomaidou
Rebecca Matsas
Socrates Tzartos
Piotr Bregestovski
Marios Zouridakis

HELLENIC PASTEUR INSTITUTE

CAPACITIES

Please send your CV and an application letter to Dr. Dimitra Thomaidou
thomaidou@pasteur.gr, no later than 30 June 2013

FP7 REGPOT 2010-1 Neurosign 264083

Theoretical and Practical workshop

"LIVE CELL IMAGING AND ELECTROPHYSIOLOGY"

Organized by:

Dimitra Thomaidou

Rebecca Matsas

Socrates Tzartos

Piotr Bregestovski

Marios Zouridakis

Preface

Dear Meeting Attendees,

We would like to welcome you to the workshop on "Live Cell Imaging and Electrophysiology", taking place in Athens between the 1st and 4th of October 2013.

The aim of this workshop is to provide post graduate and post doctoral scientists with a conceptual and practical understanding of CNS live cell imaging in whole animal level, as well as electrophysiology.

During the practicals, the participants will have the chance to observe both live and video recorded experiments including surgical procedures for creating brain and spinal cord windows for intravital Multiphoton imaging and whole-cell and single-channel recordings, using the patch-clamp technique applied on mammalian cells.

We would like to acknowledge the funding provided by the FP7 REGPOT 2010-1 EC Program "NeuroSign" that aims at the establishment of a Centre of Excellence for the study of Neurosignalling during nervous system function and dysfunction at the Hellenic Pasteur Institute.

We are looking forward to seeing you and interacting with you in this workshop and we hope that it will be an educational and enjoyable experience for everyone involved.

The Organizing Committee

"Live cell imaging and electrophysiology"

PROGRAM

1st Day, Tuesday, October 1

9:00 - 9:15 Welcome Introduction: ***Socrates Tzartos, Dimitra Thomaidou***

Chair: Marios Zouridakis

9:15-10:45 ***Piotr Bregestovski***, *University Aix-Marseille*
General principles and techniques of electrophysiological recordings; heterologous expression of ionic channels and recordings using Xenopus oocytes

10:45-11:45 ***Frank Kirchhoff***, *University of Saarland*
Differentiation of NG2 glia in health and disease

11:45-12:15 *Coffee break*

12:15 – 13:15 ***Davide Ragozzino***, *University of Rome*
The patch clamp recording in acute slices

13:15-14:00 *Lunch break*

Afternoon practical sessions – rotation mode*

14:00-18:00 **1. Electrophysiology,** Instructors : Piotr Bregestovski, Svetlana Buldakova**,** Dafni Chroni

2. Cranial window for brain imaging, Instructors : Bogdan Catalin, Paraskevi Koutsoudaki

3. Spinal cord window for SC imaging, Instructors : Dimitrios Davalos, Naoto Kawakami

4. Multiphoton imaging and analysis, , Instructors : Evangelia Xingi, Frank Kirchhoff, Dimitra Thomaidou, Paraskevi Koutsoudaki

- *The 20 students participating in practicals will be divided into 4 groups of 5 persons each. All four practicals will be performed during all the days of the workshop and each group will attend a different practical every day according to the following program:*

	GROUP 1	GROUP2	GROUP 3	GROUP 4
Tuesday 1/10	Practical 1	Practical 2	Practical 3	Practical 4
Wednesday 2/10	Practical 4	Practical 1	Practical 2	Practical 3
Thursday 3/10	Practical 3	Practical 4	Practical 1	Practical 2
Friday 4/10	Practical2	Practical 3	Practical 4	Practical 1

2nd Day, Wednesday, October 2

Chair: Rebecca Matsas

9:15-10:15 ***Naoto Kawakami***, *Max Planck, Munich*
Intravital two-photon imaging of autoreactive T cells interacting with blood-brain barrier

10:15-11:15 ***Irini Skaliora & Pavlos Rigas****, BRFAA Athens*
Simultaneous intracellular and field potential recordings of spontaneous cortical activity in brain slices

11:15-11:45 *Coffee break*

11:45 – 12:45 ***Dimitra Thomaidou***, *Hellenic Pasteur Institute*
In vitro and in vivo imaging of reactive astrocytes' dynamics

12:45-14:00 *Lunch break*

Afternoon practical sessions – rotation mode

3rd Day, Thursday, October 3

Chair: Dimitra Thomaidou

9:15-10:15 ***Rebecca Matsas,*** *Hellenic Pasteur Institute*
Stem Cells for Modeling and Treating CNS Diseases and Neurotrauma

10:15-11:15 ***Cornelia Poulopoulou****, University of Athens*
Experimental approaches for analysis of function of ionic channels and its modulation by G-proteins

11:15-11:45 *Coffee break*

11:45 – 12:45 ***Dimitris Davalos***, *University of California*
Microglia in the CNS: Challenges and lessons from in vivo imaging

12:45-14:00 *Lunch break*

Afternoon practical sessions – rotation mode

4th Day, Friday, October 4

Chair: Paraskevi Zissimopoulou

9:15-10:15 ***Frank Kirchhoff,*** *University of Saarland, Homburg, Germany*
Transgenic mice with cell-specific expression of fluorescent proteins as important tools for in vivo imaging

10:15-11:15 ***Davide Ragozzino****, University of Rome*
Recording of currents from glial cells and imaging analysis of intracellular calcium or chloride ions

11:15-11:45 *Coffee break*

11:45 – 12:45 ***Piotr Bregestovski*****,** *University Aix-Marseille*
Simultaneous monitoring of electro-physiological and fluorescent signals

12:45-14:00 *Lunch break*

Afternoon practical sessions – rotation mode

ABSTRACTS

&

OUTLINE

of

PRACTICAL TRAINING

ABSTRACTS

General principles and techniques of electrophysiological recordings; heterologous expression of ionic channels and recordings using Xenopus oocytes

Piotr Bregestovski, *University Aix-Marseille*

All biological organisms are self-assembling macromolecular systems capable to generate electrical energy. While the first evidences of Luigi Galvani on "biological electricity" (1791) were accepted with great difficulties, due to efforts of several generations of researches we know now that all cells are capable to generate resting potentials with negative intracellular sigh. Moreover, communication between cells, treatment of information and function of neuronal circuits are performed due to capability of cells to generate electrical signals. Most electrical phenomena occurring at a cell membranes are based on asymmetrical ion distributions between cytoplasm and extracellular space and on ion-selective membrane conductances. Amplitude of electrical signals is determined by function of specific proteins, primary ionic channels and pumps. Thus, every living cell represents a beautiful, munticomponent electrochemical compartment.

For electrical activity monitoring from single cells, brain slices or whole animals, a number of techniques were developed. Main of them are:

- extracellular recording;
- microelectrode intracellular recording;
- sucrose bridge technique;
- double microelectrode voltage clamp technique;
- patch-clamp technique.

To quantify and analyze the intracellular electrical processes in cells, two main strategies were proposed:

- **Current clamp** recording, allowing to determine cellular potentials, i.e. resting membrane potentials, synaptic potentials, action potentials etc.
- **Voltage clamp** recording allowing to estimate currents passing through cellular membranes, voltage-gated and receptor-operated channels.

Electrophysiological techniques have turned out to be a powerful method allowing to study processes in majority of cell types and even intracellular compartments of biological organisms. The combination of electrophysiology with molecular biology, biochemistry and cell culture allows obtaining fundamental information on structure, function and regulation of ionic channels and other charge-translocating proteins. This can be achieved by expression of genetically modified proteins in heterelogous systems like *Xenopus* oocytes or cell lines and its functional characterization by electrophysiological methods.

In the lecture practical details of electrophysiological recordings will be presented.

Differentiation of NG2 glia in health and disease

***Frank Kirchhoff**, University of Saarland*

NG2 is a type I transmembrane glycoprotein. In the central nervous system NG2- expressing cells have been identified as a novel type of glia with a strong potential to generate oligodendrocytes in the developing white matter.

For temporally-controlled gene targeting of NG2 glia in vivo, we generated a mouse line in which the open reading frame of the tamoxifen-inducible form of Cre recombinase (CreERT2) was inserted into the NG2 locus. Here, we investigated the differentiation potential of NG2 glia at different developmental stages of the forebrain. TgH(NG2-CreERT2) mice were crossbred to Rosa26-tdTomato and Rosa26-EYFP reporter lines to identify NG2 glia and its progeny.

Induction of recombination at embryonic stages revealed that embryonic NG2+ cells mainly generated more NG2 glia and oligodendrocytes, however, a significant number of astrocytes could be detected as well. Recombined cells were predominantly restricted to the ventral brain. In contrast, early postnatal injections of tamoxifen induced recombined cells that were widely found in all brain regions, thereby suggesting a distinct subpopulation of NG2+ cells after birth. When tamoxifen was injected after P8, NG2 glia stopped generating astrocytes, with the progeny largely restricted to the oligodendrocyte lineage. However, consistently, we found recombined NeuN+ cells with the morphology of neurons in the ventral cortex after administration of tamoxifen at P8. Our results suggest that NG2+ cells display a broad differentiation potential in the healthy CNS that appears to be highly age-dependent during development.

Secondary injury processes after acute brain trauma involve activation of different cell types like astrocytes, oligodendrocytes (OLG) and microglia. We were able to identify a particular type of activated glia expressing astro- AND- oligodendroglial properties simultaneously (AO cells) that transiently appeared after acute cortical injuries. AO cells could be labeled by oligodendrocyte precursor cell (OPC) markers Olig2 and Sox10, but not for markers of mature astrocytes or neurons. Two-photon live imaging revealed that AO cells originated from OLG lineage cells. Two weeks after injury, we found that the majority of AO cells became GFAP-positive, thereby providing strong evidence for OPCs giving rise to astrocytes after acute cortical injuries. To further understand the molecular mechanism, we performed intra-cerebral injection of bone morphogenetic protein 4 (BMP4, known to promote astrocyte, but blocking OLG differentiation). BMP4, but not leukemia inhibitory factor (LIF), significantly increased the number of AO cells as well as astrocytes in treated mice.

In conclusion, NG2/OPCs display strong potential to differentiate into astrocytes after acute cortical injury.

The patch clamp recording in acute slices

Davide Ragozzino, *University of Rome*

The introduction of patch clamp recording in acute slices (Edwards et al., 1989) lead to great advances in brain physiology. The strength of this technique is the possibility to record from neurons in their native environment, maintaining most of their connections unaltered

The aim of this talk is to give an overview of the techniques for preparing and performing patch clamp experiments, including:

i) methods for slices preparation from different brain areas;

ii) visualization of cells and methods for approaching and recording from neurons in acute slices

iii) advantages and applications of patch clamp recording in slices.

A second part of the talk will summarize the basic properties of synaptic physiology, emphasizing the very important achievements obtained with slice patch clamp recording: properties of evoked and spontaneous synaptic currents; the use of antagonists; the study of pre and postsynaptic mechanisms of modulation of synaptic transmission in slices; developmental studies.

The final part of the talk will focus on the different approaches used to obtain high quality slices for experimental use.

Intravital two-photon imaging of autoreactive T cells interacting with blood-brain barrier

Naoto Kawakami, *Max Planck, Munich*

In vivo imaging provides valuable information for better understanding about the physiological phenomena in the living body. Among the imaging methods, fluorescence microscopy based imaging gives good spatial and time resolution to observe cellular motility at the single cell level. Especially, two-photon microscopy is suitable for intravital imaging because of low phototoxicity and high penetration depth.

We applied two-photon intravital microscopy to observe auto-antigen specific T cells in the living animals. To this end, spinal cord of anesthetized animal was exposed surgically and animal was stabilized under microscopy. It is very important to maintain the physiological conditions of animals during entire imaging. Otherwise, the cellular response can be influenced. Therefore, we set the animal controlling system, which records the air pressure, oxygen and anesthesia gas concentration in intubation tube, Electrocardiography, oxygen saturation in the blood, and controlling body temperature by heat-pad with feedback function. All parameters are recorded and used for offline analysis.

To visualize T cells, they were labeled with GFP by using retroviral vector. This labeling allows us T cell motility but not T cell function. Recently, we introduced fluorescent protein based T cell activation sensors. One of sensor detects the intracellular Ca^{2+} by fluorescence resonance energy transfer (FRET), and another utilizes the translocation of nuclear activation factor of activated T cells (NFAT). These sensors proteins will not only visualize T cell motility but also detect T cell activation in vivo.

As results, intravital two-photon imaging showed that autoreactive T cells, at first, crawl on intraluminal surface of leptomeningeal vessels. This crawling does not induce T cell activation and is integrin alpha4 dependent. After the extravasation, T cells make contact with local antigen presenting cells antigen dependent manner and get activated.

Simultaneous intracellular and field potential recordings of spontaneous cortical activity in brain slices

***Irini Skaliora & Pavlos Rigas**, BRFAA Athens*

Brain cells do not operate in isolation but are embedded in neuronal assemblies that are characterized by coherent activity patterns in the form of oscillating activity. This is visible at increasingly macroscopic neurophysiological levels: from the local field potentials (LFPs), to the clinically relevant electrocorticography (CoG) and electroencephalography (EEG). Coherent neuronal activity in the brain is rhythmic and neuronal networks in the mammalian forebrain demonstrate several oscillatory bands covering frequencies from approximately 0.05 Hz to 500 Hz.

Originally, the knowledge and technology available limited neuroscientists to simply describe brain oscillations and correlate them to behaviour. The advent of the *in vitro* techniques enabled the study of the underlying cellular and network mechanisms under controlled conditions. The slice preparation provides a steady and easily controllable environment, which optimizes combined electrophysiological and pharmacological studies. Work in slices as early as the 1970's has brought about groundbreaking progress in our understanding of the neurobiology of pathological synchronized brain activity, such as epilepsy, while more recent in vitro research has begun to unravel the mechanisms of non-pathological cortical rhythms such as the slow oscillation, the hallmark of the quiescent states of the brain such as non-REM sleep, quite wakefulness or anesthesia.

To understand the generation and maintenance of network activity we must monitor simultaneously the activation pattern of individual cells and neuronal assemblies. In this talk we shall describe methodologies for combined extracellular LFP recordings that provide information of the coordinated activation of large groups of neurons and intracellular (whole-cell patch clamp) recordings that document the electrical behaviour of individual neurons.

In vitro and in vivo imaging of reactive astrocytes' dynamics

***Dimitra Thomaidou**, Hellenic Pasteur Institute*

Recent studies demonstrate that astroglial cells isolated from non-neurogenic brain regions have the potential to be reprogrammed into functional neurons through forced expression of transcription factors known to instruct neurogenesis. Based on our previous studies on the potential of the neurogenic gene Cend1 in directing neural stem/precursor cells (NSC) to exit the cell cycle and acquire a neuronal phenotype, in parallel with evidence demonstrating activation of Cend1 expression by genes of the neurogenin family, we explored the combined effect of Cend1 and Neurogenin-2 on their reprogramming potential on postnatal cortical astrocytes. To achieve over-expression of the two molecules we transduced astrocytic cultures with two recombinant retroviral vectors expressing Cend1 together with the green fluorescent protein or/and Neurogenin-2 together with the red fluorescent protein Ds-Red. Our results indicated that forced expression of either Cend1, Neurogenin-2 or both, resulted in an important increase of two morphologically distinct subpopulations of $GFAP^-$ cells with elongated morphology, that strongly expressed the radial glial marker glutamate transporter GLT-1. Further characterization revealed that a subpopulation of these cells differentiates towards the neuronal lineage, as they were exhibiting a differentiated neuronal morphology and expressed β-III tubulin and neuronal subtype-specific markers, including GABA and TH.

To explore whether tranduced astrocytes directly trans-differentiate to neurons or they pass through a proliferative stage, we performed long time live cell imaging experiments of astrocytic cultures lasting for 1 week. By the end of experiment, videos of astrocytes over-expressing either Cend-1 or Ngn-2, acquired from approximately 100 different areas of a 24-well plate were analyzed using 'TTT' image processing software that permits tracking of cell divisions in real time and parallel drawing of lineage tress of tracked cells. The cells that were chosen to be tracked back during their division/ differentiation route were the ones that exhibited neuronal molecular phenotype by the end of the culture. Digital image data analysis revealed that the majority of Cend1-transduced astrocytes undergo 1-2 cell divisions before differentiating to GABAergic neurons, whereas most $Ngn2^+$ astrocytes directly trans-differentiate giving rise to TH^+ neurons. Additionally, only in the double-transduced cultures, $Cend1^+/Ngn2^+$ astrocytes seemed to take a step back forming colonies of small round $Glast^+/Nestin^+$ cells, detected 24h following transduction. A day later, these colonies grew as three-dimensional spheres of high proliferative potential attached to the culture dish. When 'astrospheres' were isolated and cultured under NSC conditions, they propagated as neurospheres and, in the absence of growth factors, they differentiated into neurons, astrocytes and oligodendrocytes, implying that they possess neural stem cell properties.

In parallel studies are in progress to explore the regenerative potential of the two neurogenic genes *in vivo* in directing the reprogramming of activated astroglia following traumatic brain injury towards a neuronal phenotype. To do so we have very recently started following in real time in anaesthetized transgenic mice expressing fluorescence labeled proteins under the control of astroglia-, neuronal- and microglia-specific promoters the differentiation properties of transduced astrocytes, as well as their interaction with resident activated astroglia and microglia by 2-photon microscopy intravital imaging. To achieve forced expression of Cend1 and Ngn2 exclusively in astrocytes we have constructed astrocyte-specific lentiviral vectors expressing the two molecules. These recombinant vectors are currently stereotaxically injected in the injured cortex of hGFAP-CFP transgenic mice in order to monitor the possible astrocyte to neuron transition upon the neurogenic molecules forced expression.

Stem Cells for Modeling and Treating CNS Diseases and Neurotrauma

Rebecca Matsas, *Hellenic Pasteur Institute*

Neurodegenerative diseases and injuries of the brain or the spinal cord are characterized by loss of specialized cells of the nervous system and result in the development of defined psychiatric or neurological symptoms of varying severity, ranging from cognitive deficits and movement disorders to paralysis and death. Currently available modalities are largely ineffective for most of these situations and, therefore, the development of novel cell-based therapeutic strategies represents a serious scientific challenge with important clinical applications. Stem cells offer a unique opportunity for the development of cell-based therapies and such prospects have been reinforced since the discovery that adult human specialized cells may be reprogrammed to a pluripotent embryonic stem cell-like state. We have been using neural stem cells, naïve or genetically modified with therapeutic molecules, to assess their regenerative potential after transplantation in various models of brain injury. We observed that neural stem cell transplantation is effective in restoring learning and memory decline in hippocampal injury paradigms, as assessed by exposure of the injured animals to the Morris Water Maze task. At the histological level, neural stem cell transplantation protected against neurodegeneration and enhanced regenerative cellular processes. These data highlight the prospective Translational Medicine potential of neural stem cell grafting for treating CNS disease and neurotrauma. More recently, we have been engaged in the generation of iPS cells from human fibroblasts from Parkinsonian patients and unaffected individuals. We have obtained iPS clones from two Parkinsonian patients with a genetic form of the disease as well as from age-matched healthy individuals. iPS-derived dopaminergic neurons were obtained by directed differentiation of iPS cells and their morphological, biochemical and electrophysiological properties are being analyzed. We anticipate that this disease-in-a-dish cellular model should be useful for studying the pathophysiology of Parkinson's disease as well as for drug screening and discovery.

Experimental approaches for analysis of function of ionic channels and its modulation by G-proteins

***Cornelia Poulopoulou**, University of Athens*

The development of the patch clamp technique provided electrophysiologists with the unprecedented opportunity to directly record with high resolution, ionic currents flowing in or out of the cell membrane. The technique allowed the identification and characterization of a large number of different native ion channels and has provided new insights into the role of ion channels in an array of cellular functions and thus in cell physiology and patho-physiology. Ion channels are present in all living cells and are categorized by their ion selectivity (K+, Na+, Ca++, Cl-) and their gating mechanism (voltage and ligand gated). The intrinsic biophysical properties of a channel are solely determined by the subunit composition of the channel-protein and are invariables. When the intrinsic properties of a channel are altered through a biochemical modification of the channel-protein i.e. phosphorylation, we talk about modulation of the channel. Employment of the patch clamp technique apart from enabling us to characterize with high accuracy the structural and biophysical properties of the different ion channels, allows us to study in a direct and functional manner the modulation (alteration) of the channel activity by G-protein linked or growth factor and hormone receptors.

In this presentation we are going to concentrate on the study of ion channel modulation and in particular "the modulation of the voltage-gated ion channels by G-protein coupled receptors" using the patch clamp technique. To this end we are going to discuss the design of experimental protocols that would allow the identification of the intracellular pathways and cellular components responsible for the alterations of the biophysical parameters of a particular channel and thus delineate the molecular mechanism involved in the modulation of this channel. Furthermore we are going to show how the activation of a G-protein coupled receptor may affect the voltage-dependence, the speed of gating, and the probability of opening or closing of an ion channel by presenting whole-cell currents and single channel recordings from experiments and published data. As examples for the study of voltage-gated channel modulation by G-proteins we are going to use the data and the experimental procedures from a) the modulation of a voltage-gated Ca++ channel by the β-adrenergic receptors and b) the modulation of a voltage-gated K+ channel by the metabotropic glutamate receptors. At this point it is worth mentioning that channel modulation through receptor activation seems to ensue adaptation of the activity of the channel to the spatiotemporal needs of the cell.

Microglia in the CNS: Challenges and lessons from in vivo imaging

Dimitris Davalos, *University of California*

Microglia form the first line of defense against injury or disease for the central nervous system (CNS). Microglia were long believed to remain in a dormant/resting state under physiological conditions and be activated only by an insult to the brain. Using two-photon microscopy, we imaged microglia in vivo and revealed, for the first time, a new function for microglia: they continuously screen the intact brain parenchyma with their fine-processes on a timescale of minutes[1]. When challenged with a focal injury model, microglia also rapidly respond, by extending their processes and isolating the injury, thereby protecting the surrounding healthy tissue[1]. In neurodegenerative or neuroinflammatory diseases, microglial responses range from protective to harmful. Over the past decade, in vivo imaging in transgenic mice with fluorescently labeled microglia has essentially redefined our understanding of microglial functions in the brain, in health and disease. However, breathing-induced movement artifacts had for a long time impeded the application of in vivo imaging in studies of the spinal cord, the other major CNS site with relevance for traumatic injury and neuroinflammatory disease. By developing a novel imaging method for stabilizing the mouse spinal column, we overcame these technical limitations and were able to extend the application of this powerful tool to studying the living spinal cord[2,3]. Using this method, we performed longitudinal in vivo imaging of microglial responses in the mouse spinal cord, throughout the course of neuroinflammatory disease similar to multiple sclerosis (MS). We showed that microglia respond rapidly to sites of disruption of the blood brain barrier by forming perivascular clusters where the blood factor fibrinogen leaks and is deposited in the spinal cord parenchyma[4]. We also found that these microglial clusters mark the areas of axonal damage, and that fibrinogen is required to activate microglia to release reactive oxygen species and directly contribute to axonal damage in MS-like lesions[4].

1. Davalos, D*., et al.* ATP mediates rapid microglial response to local brain injury in vivo. *Nat Neurosci* 8, 752-758 (2005).
2. Davalos, D*., et al.* Stable in vivo imaging of densely populated glia, axons and blood vessels in the mouse spinal cord using two-photon microscopy. *J Neurosci Methods* 169, 1-7 (2008).
3. Davalos, D. & Akassoglou, K. In vivo imaging of the mouse spinal cord using two-photon microscopy. *J Vis Exp*, e2760 (2012).
4. Davalos, D*., et al.* Fibrinogen-induced perivascular microglial clustering is required for the development of axonal damage in neuroinflammation. *Nat Commun* 3, 1227 (2012).

Transgenic mice with cell-specific expression of fluorescent proteins as important tools for in vivo imaging

Frank Kirchhoff, *University of Saarland, Homburg, Germany*

Genetically modified mice with cell-specific expression of fluorescent proteins have become an important tool to investigate a variety of cell functions in vivo.

Here, I will provide a comparative analysis and description of various transgenic mouse lines with fluorescent protein (FP) expression in the central and peripheral nervous system. In particular, I will discuss the expression properties of FPs in different glial cell types in grey and white matter such as astroglia, oligodendroglia, NG2 glia and microglia. Furthermore, the technical advantages and difficulties of transgenic mice generated by different non-homologous and homologous recombination strategies will be highlighted. This will also include the use of tamoxifen-induced gene recombination to achieve cell labeling, but also the induction of genetically encoded calcium indicators.

Recording of currents from glial cells and imaging analysis of intracellular calcium or chloride ions

***Davide Ragozzino**, University of Rome*

In this talk, the different types of glial cells, their main roles and the possible influences they exert on synaptic transmission will be described, with particular attention to microglia cells, the methods to record from microglial in brain slices and the typical electrophysiological properties of microglia cell in slice or cell cultures.

The second part of the talk will be dedicated to the methods for dynamic measurement of intracellular ion movements. After a short introduction about the basics of fluorescence measurements, we will analyse ii) the use of fluorescent dyes for intracellular Ca^{2+} monitoring; iii) the different approaches to study calcium permeability of ligand gated channels. The final part of the talk will be focused on the methods and problems linked to the study of intracellular chloride; in particular, i) the preservation of Cl^- equilibrium and its relevance in inhibitory signaling in neurons; ii) the use of non invasive fluorescent methods for intracellular Cl^- monitoring and it applications.

Simultaneous monitoring of electro-physiological and fluorescent signals

Piotr Bregestovski**,** *University Aix-Marseille*

Simultaneous monitoring of both the ionic currents and light signals from fluorescent molecules become powerful tool for investigation function of various proteins, as well as physiological properties of cells, neuronal circuits and biological organisms.

Optical stimulation of electrically excitable cells is superior to classical activation by microelectrodes because of the high temporal and spatial resolution. Light activation of neural cells can be achieved by using caged compounds, for example caged ATP, GABA or glutamate, whereby the substrates for activation of ion channels are delivered by light from the chemical photolabile cage in the microsecond and millisecond time scale. Development of probes for non-invasive monitoring of ions or fluorescently labeled ionic channels provides excellent tools for visualization and analysis of neuronal function in normal and pathological conditions.

An improved approach is the genetical encoding of the activator in the target cell or the application of chemical photo-switches attached to ion channels, which allows to depolarize cells reversibly with high temporal and spatial resolution. These elegant techniques are applicable for neurons in culture as well as in small animals like drosophila and zebrafish.

Optogenetical approach become a new highly promising direction of research, which allows non-invasive control of different neuronal subsets or functional analysis of proteins, like ionic channels. The discovery of Channelrhodopsins (ChR1 and ChR2) from the unicellular alga *Chlamydomonas reinhardtii* was the starting point for the optogenetic. When transfected into mammalian cells and activated by blue light, ChR2 acts as cation channel, thus depolarizing the cells. Together with the yellow light-activated hyperpolarizing Cl-pump Halorhodopsin from the archaeum *Natronomonas pharaonis* (NpHR), the two microbial rhodopsins form an ideal pair for the activation and inactivation of cells.

The growth of optogenetic methodologies has included the advancement of several approaches:

(1) one- and two-photon fluorescence microendoscopy for imaging fluorescently labeled cells deep within the mammalian brain;

(2) simultaneous imaging of mammalian functional brain maps and the dynamics of neuronal morphology in genetically targeted neocortical cells;

(3) genetically targeted reporters for imaging subcellular biochemical function in living neurons;

(4) genetically targeted optical control of neural activity in behaving *Drosophila* and electrical activity in mammalian circuits.

Resent observations and application of new tools for molecular imaging and remote activation of receptors, ionic channels and synaptic networks will be presented.

PRACTICAL 1

Electrophysiology: Patch-clamp recording from CHO cells transiently expressing glycine receptor channels

Outline for the practical session

Piotr Bregestovski, Svetlana Buldakova, Dafni Chroni

General aim.

To obtain practical knowledge on the expression of foreign proteins in cultured cells and on patch-clamp recording of ionic currents.

Specific aim.

With the help of the teacher:
- to obtain different patch clamp configurations;
- to record the dose-response curve for glycine
- to obtain current-voltage relations for glycine-induced currents.

Experimental model:

CHO cells in a culture transiently expressing Cl-selective glycine receptor channels.

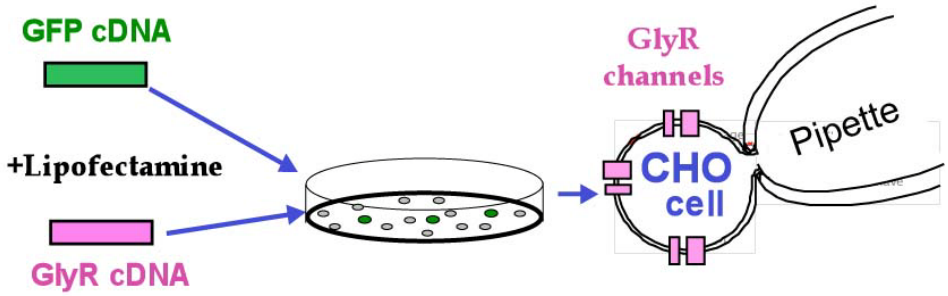

Fig.1. Scheme of expression of glycine receptor (GlyR) channels in CHO cells.

Tasks:

To perform CHO cells culture and expression of GlyR channels:

- how to make passages of cells;
- how to put cultured CHO cells on cover glasses;
- how to perform transfection of CHO cells using the Lipofectamin transfection protocol;

For electrophysiological recording:

A. Set up, reference electrode and pipette holder:

- basic organization of set up:
  - antivibration table;
  - microscope;
  - fluorescent illumination;
  - manipulators;
  - fast perfusion system;
  - suction system;
- basic principles of HEKA amplifier functioning;
- Patch-Master program, main functions;
- how to neutralize junction potentials:
  - "chloriding" of reference electrode and pipette holder;
  - neutralizing using HEKA-9 amplifier;

B. Solutions and perfusion:

- how to make an external solution;
-how to make an internal solution;
- how to filtrate solutions;
- how to prepare different concentrations of the agonist (glycine);
- how to fill the fast perfusion system:
  - to avoid bubbles;
  - to achieve equal speed of perfusion from different tubes;

C. Pipettes

- how to make pipettes for patch-clamp recordings;
- how to fill up pipettes with internal solution;

D. Performing of the experiment:

- how to approach pipettes to cells:
  - positive pressure in the pipette;
  - electrical and visual control;
- how to obtain a cell-attached configuration;
- how to obtain a whole-cell configuration;
- how to approach fast perfusion tubes;
- how to estimate the input resistance of the cell;
- how to estimate the resting potential of the cell;
- how to obtain concentration dependence for glycine;
- how to obtain current-voltage relations;

E. Data analysis:

- how to transfer data from Patch-Master program to "Origin" or "Igor";
- how to make a graph of the concentration dependency;
- what information can be obtained from the graph;
- how to make a graph of current-voltage (I-V) dependency;
- what information can be obtained from the I-V graph.

Basic information on patch-clamp recording

The patch-clamp recording is an extremely powerful technique, which revolutionized our

electrophysiological analysis of various cells of biological organisms. Soon after the development by Erwin Neher and Bert Sakmann (1976-1981) it was adopted by numerous laboratories and caused a tremendous advancement of many research areas in both cellular and molecular biology.

What is patch clamping?

The patch-clamp technique is an electrophysiological method that allows the recording of macroscopic whole-cell or microscopic single-channel currents flowing across biological membranes due to activation of ion channels. Active transporters may also be studied in cases where they produce measurable currents, i.e. they are not electroneutral (e.g. Na^{+}-Ca^{2+} exchanger or amino acid transporters).

Patch-clamp means imposing a defined voltage on a small membrane patch with the purpose to measure the resulting current and consequently the calculation of the patch conductance. Originally, the patch-clamp technique referred to voltage-clamp and current-clamp measurements using "patch-clamp"-type micropipettes.

For the first time Neher and Sakmann applied this technique in 1976 to record the tiny (pico-Ampere, pA, pico = 10^{-12}) ion currents through single acetylcholine-activated channels in the frog neuro-muscular junction. At that time it was an almost unbelievable achievement, which was later awarded the Nobel Prize (1991).

Five main configurations of patch-clamp recording:

1. Cell-attached. Using pipettes filled with filtered solution and allying of slight positive-pressure to the pipettes allows to obtain super-clean tips during the approach to the cell. This procedure caused tight sealing of the membrane against the pipette tip with seal resistance of several Giga-Ohm (Giga=10^{9}). This measurement configuration is called cell-attached patch (CAP) (see Fig. 1). It allows the recording of single-channel currents from the sealed patch with the intact cell still attached.

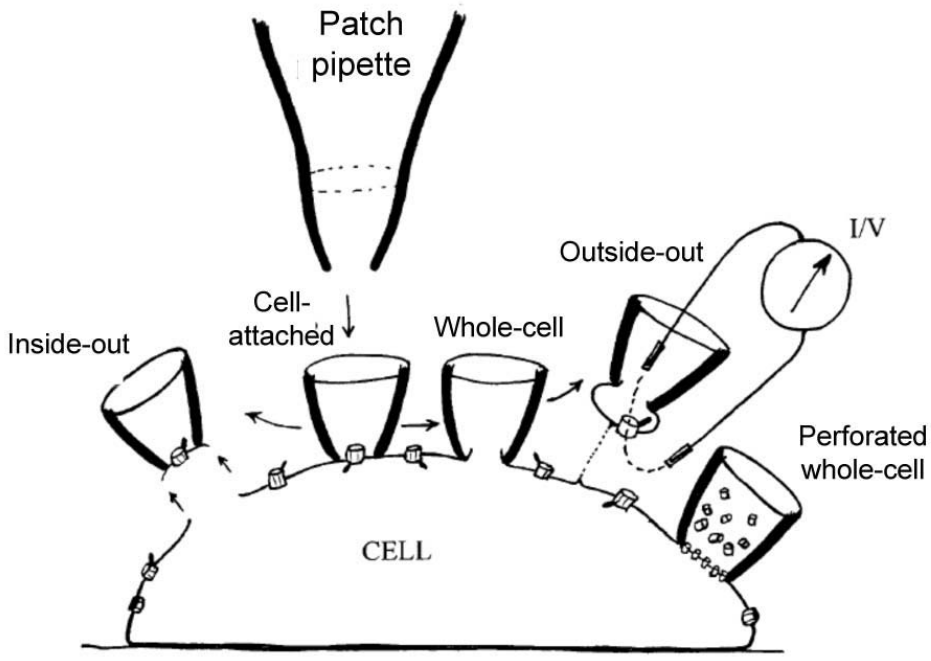

Fig. 2. Main configurations of patch-clap recording

2. Whole-cell. The configuration when the membrane patch between the pipette solution and the cytoplasm is gently broken by a suction pulse while maintaining the tight seal is called whole-cell recording (Fig.2). It allows estimation of resting membrane potential, input resistance of the cell, recording ionic

currents induced by activation of voltage-gated and receptor-operated channels etc. This configuration also allows changing intracellular ionic composition by perfusing the intracellular compartment with the defined pipette solution.

3. Inside-out configuration. The excised patch is called an inside-out configuration (Fig. 2), because the inside of the plasma membrane is exposed to the external salt solution. This configuration allows to exposure the cytoplasmic side to defined solutions in order, for example, to test the intracellular factors that control membrane channel activity.

4. Outside-out configuration. This type of excised patch can be obtained, but from the whole-cell configuration by slowly (not abruptly!) pulling the pipette away from the whole cells (Fig. 1). This maneuver first defines a thin fiber that eventually breaks to form a vesicle at the tip of the pipette. This configuration is actually a micro-whole-cell configuration, which allows one to study small populations of channels or single channels and to readily manipulate the "tiny cell" to different bathing solutions for rapid perfusion.

5. **Perforated (permezabilized) whole-cell configuration.** Here cell-attached patch is not ruptured for direct access to the inside of the cell, but made permeable by adding artificial ion channels (monovalent cation channel-forming antibiotics) via the pipette solution (Horn and Marty, 1988). Examples of such antibiotics are amphotericin and nystatin, both produced by microorganisms. The great advantage of this configuration is that it allows intracellular voltage and current-clamp measurements on relatively intact cells, i.e. cells with a near normal cytoplasmic composition.

Patch-clamp set-up.

The main components of the patch-clamp set up are: t**he cell chamber** on the stage of **the microscope** with the small **patch-clamp preamplifier box** (probe or head-stage) above the cells. The head-stage is held and manipulated by a **micromanipulator** and has a connector for connecting **the pipette-holder**. All this is standing on the **vibration isolation table,** and **Faraday cage** is used to prevent external electrical noise. Also the **patch-clamp amplifier**, **computer** with **specific interface** and **programs** to control the amplifier, as well as data acquisition, storage and data analysis.

Necessary additional equipment and components:

- **pipette puller** for fabrication parch recording pipettes.
- **pipette microforge** is useul to check quality of some pipettes during fabrication and to polish them, as this facilitate obtaining of gigaseal contacts.
- **pipette holder** made from Teflon or polycarbonate to have low dielectric loss. The pipette electrode is simply a thin silver wire (3-5 cm long) that is soldered onto the pin that plugs into the connector of head-stage preamplifier. The silver electrode should be chlorided along its entire length to obtain low junction potential and to avoid significant drift of the pipette potential.
- **reference electrode**. The main requirements for a bath electrode are stability and reproducibility of the electrode potential. In simplest case, the reference electrode can be made from a peace of silver wire with a diameter of 0.5-1 mm. If cells are intolerant to silver, an agar salt bridge is advisable (U-tube capillary filled with agar). The agar can be made up on the bath solution.

Preparation of solutions:

Firstly stock solutions should be prepared of the following concentrations and quantities:

5M **NaCl** - 146 gr / 500 ml H_2O
1M **KCl** - 7.45 gr/ 100 ml H_2O
1M **$CaCl_2 \times 2H_2O$** - 14.7 gr/ 100 ml H_2O

1M **MgCl2 x 6H2O** - 20,3 gr/ 100 ml H2O
0.5M **HEPES acid** - 59.6 gr/ 500 ml H2O **(pH=7.4 with NaOH)**

From these stock solutions the external solution can be prepared. Below the concentrations and quantities for preparing 1L of external solution for recording from cells in culture (CHO, HEK 293 etc) are listed.

Extracellular solution (**1 L**):

140 mM	NaCl -	28 ml (5M)
2.8 mM	KCl -	2.8 ml (1M)
2 mM	CaCl2 -	2 ml (1M)
4 mM	MgCl2 -	4 ml (1M)
20mM	HEPES/NaOH -	40 ml (0.5 M)
10 mM	Glucose -	1.98 g

pH=7.4-7.5, Osm=320-330

Depending on experimental tasks different intracellular solutions can be used. The most popular are: Cs-based for inhibiting K channels and increase input resistance of recording cells and KCl or Kgluconate solutions to prevent K conductance and/or to keep low intracellular concentration of Cl (which is usually low in neurons and other cell types).

Intracellular solution, Cs-based:

CsCl	*140 mM*
$CaCl_2$	*1.5 mM (at using EGTA $CaCl_2$ should be 1.3 mM)*
$MgCl_2$	*2 mM*
HEPES/CsOH	*10 mM*
BAPTA or EGTA/CsOH	5 *mM*
MgATP	*2 mM*
Na_2GTP	*0.4 mM*

pH=7.2 - 7.3, Osm=280-290
In this solution Ca-free concentration will be about 60 nM

Intracellular solution, K-based:

KCl or KGluconate	*140 mM*
$CaCl_2$	*1.5 mM (at using EGTA $CaCl_2$ should be 1.3 mM)*
$MgCl_2$	*2 mM*
HEPES/CsOH	*10 mM*
EGTA or BAPTA/CsOH	5 *mM*
MgATP	*2 mM*
Na_2GTP	*0.4 mM*

pH=7.2 - 7.3, Osm=280-290
In this solution Ca-free concentration will be about 60 nM

Important! After the adjustment of pH and osmolarity, the solution **should be filtered** using the filter for culture (0.2µm). Then it **should be aliquoted** in 1ml Eppendorf tubes, labelled, put in the box and put **in the refrigerator** (-20 - 80°C). Filtering will ensure a clean tip of recording pipettes and consequently - reliable Giga-seals, while freezing will strongly slowdown degradation of ATP and GTP.

Cell culture and expression of GlyR channels in CHO cells:

- how to make passages of cells (splitting);
- how to put cultured cells on cover glasses (∅10-14 mm);
- how to perform transfection of cells using Lipofectamin transfection protocol.

Solutions and materials that you will need for splitting and transfection:

Gibco F12-HAM Ref. 21765-029 Invitrogen
GIBCO L-glutamine 200 mM 100 ml Ref 25030-024
GIBCO Penicillin Streptomycin 15140-122 Invitrogen
GIBCO Bovine Serum Invitrogen
GIBCO DPBS ref 14190-094 500 ml Invitrogen
GIBCO trypsin EDTA x1 in Ref 25300-054 100 ml Invitrogen
GIBCO Optimem gluta max ref. 51985-026 Invitrogen
Lipofectamine 2000 Invitrogen 50470
Millipore filters 0.22
Petry dishes 35 mm
Cover glasses 12 or 14 mm
Flasks (Nunc) 75 cm^2 treated flask blue filter cap

Preparation of culture medium for CHO cells:

5 ml bovine serum (defrosted and warmed) (GIBCO)
500 µL Penicillin Streptomycin
500 µL L-glutamine 200 mM
44 mL F12 (fill up tube to 50)
Altogether you will have 50 ml of the medium
Filter with 0.22 µm
Put back in the fridge for later use.

Splitting protocol:

1) heat up the culture medium, PBS solution and trypsin (30 min, 37C);
2) take out all the old solution from the flask or dish;
3) rinse a flask with 4 ml of PBS (to prevent trypsin blockage);
4) take it out right away;
5) add 2 ml of trypsin EDTA in a flask and spread it out in it so that the whole flask is covered (for cells dissociation);
6) put a flask for 1 min into the incubator;
7) take it out of the incubator, close the lid tightly, and tap the bottom briskly (that will facilitate cell dissociation);
8) then add 4 mL culture medium (for trypsin blockage);
9) mix gently by suction and putting back the culture medium using 5 mL pipette for about 10 times (try not to make bubbles);
10) put 500 µL of solution with the cells in a new large flask or 250 µL in a small flask or large Petri dish;

11) then add 6 mL of CHO medium in the new flask (3 mL for small flask or large Petri dish);
12) mix by moving the flask gently;
13) put a new flask in the incubator; check after 2 days if the cells have attached to flask bottom; next splitting will be necessary when 80% of a flask bottom are covered with cells (near one week after the first splitting);
14) cells for patch clamp experiments should be grown on cover slips (∅12-14 mm). For this put 3 cover slips into a 35 mm Petri dish, take 200-300 µL of the solution with cells and put one drop of this solution on each cover glass;
15) then add 2 mL of CHO medium in a dish, mix by moving the dish gently;
16) on the next day you can make a transfection.

Transfection:

<u>You will need:</u>
Filtered (0.22µm) Optimem gluta max (no need to warm it up).
One eppendorf tube (1 mL).
Lipofectamine 2000.
Warmed CHO medium (that has been previously filtered).
For a single transfection (one 35mm Petri dish) you have to mix in the eppendorf tube: 250 microl Optimem gluta max;
1-2 µL cDNA (concentration 1-2 g/µL);
0.5 µL GFP construct (1 g/µL);
6 µL Lipofectamine 2000.

Slightly vortex a tube with this mixture.
Wait for 15 min.
Take a Petri dish with CHO cells cultured on cover glasses out of incubator.
Then transfer contents of the tube to a dish with cells, and put back a dish into the incubator.
Wait for 3 hours.
After 3 hours change a solution in the dish on the new warmed CHO medium.
Put the dish back into the incubator.
After 24 hours cells express channels and are ready for the electrophysiological experiment.
Cells can be used up to 72 hours after transfection.

PRACTICAL 2

Cranial window for brain imaging

Outline for the practical session

Bogdan Catalin, Paraskevi Koutsoudaki

The purpose of this practical is to exhibit one of the available ways of performing a craniotomy and building a cranial window on the head of a mouse, hoping to perform a long-lasting "clear passage" to the brain surface, and be able to perform long-term multiphoton imaging of the adult cortex.

1. Anesthesia (120mg/kg Ketamine HCl, 12mg/kg Xylazine HCl), intraperitoneally. Additional anesthesia if necessary.
2. Preparation of the metal part to be needed for attaching the mouse to the microscope-specific table (paper clip!).
3. Beginning the operation: removal of the skin. Extensive cleaning of the scull surface.
4. Drilling through the scull, craniotomy. Attention not to disturb the dura matter of the brain surface.
5. After removing part of the scull, extensive rinsing of the brain surface and stopping of any bleeding that might occur.
6. – Inject – Cells, Viruses, Fluorescent Dyes, last chance!!!
7. Put a drop of 1% agar on the exposed brain surface.
8. Adjust round 5mm coverslip on the exposed brain. Find the best possible orientation!
9. Glue the coverslip down on the scull using plain superglue.
10. Position the metal part on your "building site" after finding an appropriate angle.
11. Cement your window, metal part included, down to the scull of the mouse using dental cement.
12. Place the mouse on a heat pad for recovery (if imaging is not to take place immediately). Inject analgesic medication intraperitoneally.

<u>Anesthesia Recipe:</u>

3.5ml of Ketamine HCl 100mg/ml

1.5m of Xylazine HCl 20mg/ml

in 10ml NaCl 0.9%

I.P.

<u>Rinsing/Viewing Buffer Recipe:</u>

1000 Units/ml Penicillin

1mg/ml Streptomycin

in NaCl 0.9%

Analgesia:

Dexamethazone (0.2 mg/kg)

Carprofen (5 mg/kg)

in NaCl 0.9%

S.C. or I.P.

Some Fluorescent Dyes to use:

- Sulforhodamine 101 (Staining of blood vessels and astrocytes)
- Thioflavin T or Thioflavin S (Staining of amyloid plaques)
- Texas Red Hydrazide (Staining of astrocytes - fixation)
- FITC dextran (Staining of blood vessels)

PRACTICAL 3

Spinal cord window for SC imaging

Outline for the practical session

Dimitrios Davalos, Naoto Kawakami

In vivo imaging using two-photon microscopy in mice that have been genetically engineered to express fluorescent proteins in specific cell types has significantly broadened our knowledge of physiological and pathological processes in numerous tissues in vivo. The broad application of in vivo imaging in the brain has produced a plethora of novel and often unexpected findings about the behavior of cells such as neurons, astrocytes, microglia, under physiological or pathological conditions. However, mostly technical complications had for a long time limited the implementation of in vivo imaging in studies of the living mouse spinal cord. In particular, the anatomical proximity of the spinal cord to the lungs and heart generates significant movement artifact that makes imaging the living spinal cord a challenging task. We developed a novel method that overcomes these limitations by combining a customized spinal stabilization device with a method of deep anesthesia, resulting in a significant reduction of respiratory-induced movements[1].

In this practical session we will demonstrate how to expose a small area of the living spinal cord that can be maintained under stable physiological conditions over extended periods of time by keeping tissue injury and bleeding to a minimum[2]. We will show representative raw images acquired in vivo that detail the close relationship between microglia and the vasculature and a timelapse sequence of the dynamic behavior of microglial processes in the living mouse spinal cord[1]. Moreover, we will show a continuous scan of the same z-frame demonstrating the outstanding stability that this method can achieve to generate stacks of images and/or timelapse movies that do not require image alignment post-acquisition[1]. Finally, we will show how this method can be used to revisit and reimage the same area of the spinal cord at later timepoints, allowing for longitudinal studies of ongoing physiological or pathological processes in vivo[1,2].

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2. Davalos, D. & Akassoglou, K. In vivo imaging of the mouse spinal cord using two-photon microscopy. *J Vis Exp*, e2760 (2012).

PRACTICAL 4

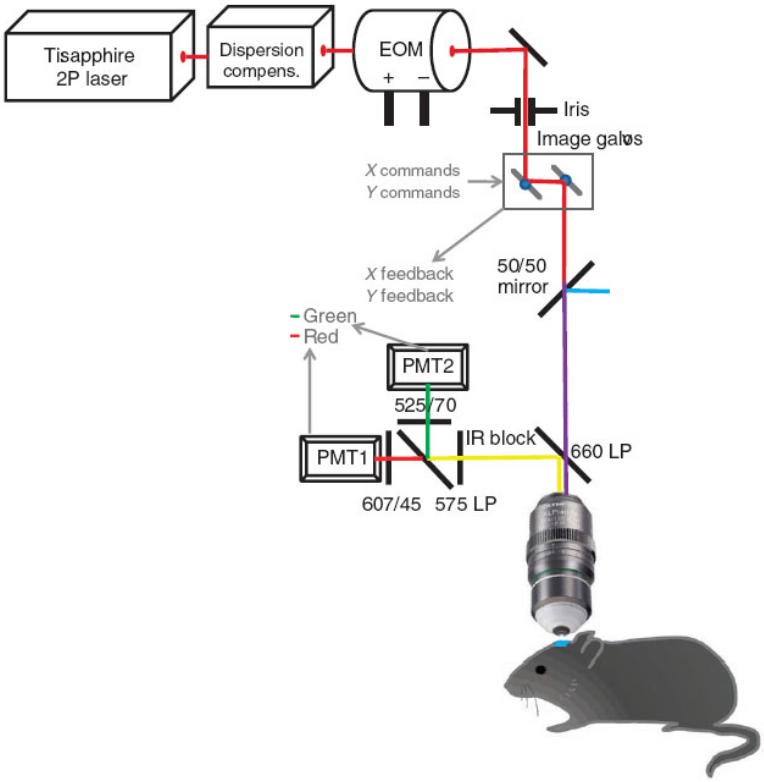
Multiphoton imaging and analysis

Outline for the practical session

Frank Kirchhoff, Evangelia Xingi, Paraskevi Koutsoudaki , Dimitra Thomaidou,

Introduction

Two-photon microscopy permits *in vivo* visualization of structures lying deep inside tissues, due to its special features including lower light scattering, restricted excitation and bleaching only at the focal plane, and reduced phototoxicity. Two-photon imaging allows monitoring of neuronal dynamics, dynamics of various types of glial cells, blood cell flow, neurovascular coupling, and cell metabolism. Two-photon imaging is applicable to a variety of mouse models of brain disease or spinal cord injury and can help to reveal mechanisms underlying pathological events.



Schematic representation of a two-photon microscope setup for *in vivo* imaging in the brain. A two-photon (2P) laser is passed through a dispersion compensation device, and then through an intensity modulator (e.g., EOM, electro-optical modulator). The laser is then steered with a pair of *X,Y* mirrors (galvanometers, 'galvos'). Light passes through the microscope, directed by the controlled mirrors as shown, and exits the objective into the brain tissue. Fluorescence can be detected in multiple color channels using photomultiplier tubes (PMTs), which can be either external or internal.

The key difference of two-photon laser scanning microscopy (TPLSM), as compared to confocal laser scanning microscopy, has to do with the physical properties of light. In a two-photon process, two photons are

absorbed virtually simultaneously (within <1 fs). Because the two photons combine their energies to promote the molecular transition, their individual energies are relatively low. Hence, excitation light is of longer wavelength compared to single-photon absorption, typically in the near-infrared wavelength range (700–1000 nm).

The use of near-infrared light is the first key advantage of two-photon excitation because longer-wavelength light is less scattered in biological tissue, enabling larger penetration depths. The second important feature is that **the fluorescence signal depends nonlinearly on excitation light intensity** (on the square of the intensity in the case of two-photon excitation). This nonlinear dependence (in defining TPLSM as a "nonlinear microscopy technique") is highly beneficial because it confines fluorescence generation to the focus spot. As a consequence, at each point in time fluorescence photons are generated only locally, and they can be correctly assigned to their point of origin in three-dimensional (3D) space irrespective of whether they are scattered on their way out of the tissue to the detector. In TPLSM a confocal pinhole in front of the detector is not necessary to achieve optical sectioning; it should be omitted and as-many-as-possible fluorescence photons should be collected.

With a TPLSM setup one can also take advantage of another non-linear optical effect, second-harmonic generation (SHG). SHG-signal is generated from highly ordered structures and has been used to image collagen fibres, microtubules, the striation pattern of muscles and starch granules, but also to measure membrane potential with SHG-suitable dyes.

In summary, the longer-wavelength excitation and the intrinsic optical sectioning property make TPLSM less sensitive to light scattering compared to confocal microscopy, clearing the view deep into the living tissue.

The procedure

During this practical 2-photon mouse brain imaging will be performed through a cranial window. The practical session will start with a short introduction on 2-photon microscopy.

Reagents

- EGFP-CX3CR1 transgenic mice expressing EGFP in microglial cells, or wild type mice, both groups bearing chronic cranial windows.
- Anesthesia: ketamine HCl, xylazine HCl in 0.9% NaCl solution.
- Sevoflurane, applied through a vaporizer, vaporized in pure oxygen.
- Eye ointment (e.g., Bepanthol).
- Sulforhodamine 101 (SR101; Sigma-Aldrich), a specific astroglial marker (Nimmerjahn et al. 2004, Appaix et al 2012). SR101 is dissolved in 0.9% NaCl (saline) at a concentration of 10mg/ml.
- FITC-dextran (70 kDa, Sigma-Aldrich) for imaging blood vessels. FITC-dextran is diluted at 100mg/ml concentration in saline.

Equipment

- Anesthesia unit including a flow meter and a vaporizer.
- System for monitoring body temperature through a rectal probe.
- Heat pad.
- Imaging setup.

A LEICA TCS SP5 two-photon imaging system will be used. It is equipped with a mode-locked Ti:sapphire oscillating laser with automated dispersion compensation (Mai Tai HP DeepSee, Spectra-Physics) and a laser-scanning system coupled to a fixed stage upright microscope. The system is also equipped with a custom-made multi-axis mouse holder stage provided by the group of Dr. Kirchhoff.

Two-photon imaging is performed with a 25×, 0.95-numerical aperture (NA) ceramic water-immersion objective, working distance 2.5 mm (HCX IRAPO L 25×, Leica). The objective has a resolution of 205 nm in xy

and 550 nm in the z-axis. Maximum resolution of the pixel size is around 100nm in the xy and 275 nm in the z-axis (1/2 of the resolution of the objective).

Fluorescence emission can be collected either with non-descanned PMTs (NDDs) or with the internal PMTs (with pinhole wide open). The use of non-descanned PMTs is recommended, as this geometry improves signal collection. The system is equipped with two NDD detectors placed near the objective. The available filters are DAPI (460/50nm), TRITC (585/40nm), FITC (525/50nm). The fluorescence is separated using dichroic mirrors (beam splitters). The mirrors used to separate DAPI/TRITC and FITC/TRITC configurations are 495 nm and 560 nm respectively. Second harmonic imaging is performed using the DAPI filter.

For simultaneous visualization of second harmonic and red dyes, the DAPI/TRITC configuration is used. The excitation wavelength used is 900 nm and the emitted light is separated at 495nm using a beam splitter, as described.

- Image analysis software: The collected images are analyzed with the LAS AF and/or the Imaris (Bitplane) software.

Procedure

1. Anesthetize the animal

Mice are weighted and initially anesthetized intra-peritoneally with 120 mg ketamine HCl, 15 mg xylazine HCl per kg in 0.9% NaCl solution (dose of 100 ul /20g of body weight). Wait for the anesthetic to take effect (approximately 10 minutes). Toe pinch withdrawal reflex is tested to ascertain that the desired level of anesthesia has been reached.

Mice are kept anesthetized for the duration of the imaging experiments either with hourly injections of half the initial dose or using sevoflurane (1-3%) in pure oxygen.

2. Monitor body temperature

The mice body temperature is monitored continuously with a rectal probe and maintained at 34°C using a heating pad placed on the mouse holder stage. To prevent the animal's eyes from drying out, eye ointment is applied.

3. Tail injection with Sulforhodamine 101 and/or FITC-dextran

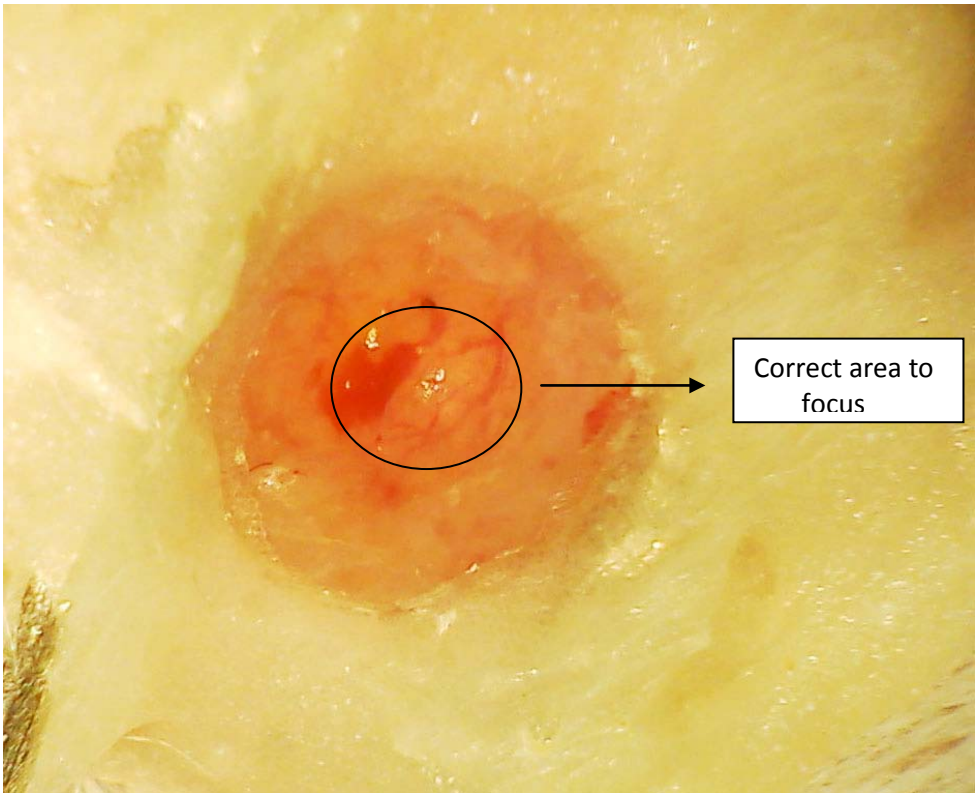
Mice are injected with SR101 (20mg/kg) and/or FITC-dextran (200mg/kg) in the tail vein. Two photon excitation wavelength is 900 nm for SR101 and 940nm for FITC-dextran. Fluorescence emission is detected simultaneously by two NDD PMTs with a TRITC (585/40 nm) filter for "red" fluorescence emission and a FITC (525/50nm) filter for "green" fluorescence emission.

4. Stabilization of mouse brain on the mouse holder stage

A mouse holder stage that could fit on a lowered microscope stage was built using a steel base plate to support the animal and a head holding adaptor. The animal is stabilized by positioning the metal clip of the brain window in the head holding adaptor of the stage.

5. In vivo imaging of the mouse brain through the cranial window

Imaging is performed using the equipment described above. Focusing in the level of the pial surface is performed through second harmonic imaging, since a camera that would help us to choose the correct region to focus is not currently available.



(a) Second harmonic imaging

Mai-Tai IR laser is tuned at 900 nm for second harmonic imaging in order to image the brain pial surface (collagen fibres). The laser is adjusted/attenuated in two ways: the "Trans"-slider, which controls the half wave plate, and the "Gain", "Offset" and "MP" sliders, which control the EOM. Second harmonic is detected either using the external NDD2 detector (DAPI filter) or the internal PMT (detection width 410-480nm). A 3D second harmonic stack can be acquired using "Z Wide", which moves the objective for focusing.

(b) Imaging of EGFP microglial cells and/or SR101 stained astrocytes and/or FITC-dextran stained vasculature.

In order to monitor the eGFP microglial cells and/or the astrocytes stained with SR101 and/or the blood vessels stained with FITC-dextran, imaging should be continued in more depth. Mai-Tai IR laser is tuned at 900 nm for either eGFP microglial cell and/or SR101 astrocyte imaging. Fluorescence emissions can be detected simultaneously by the two NDDs with the FITC/TRITC configuration. Fluorescence emissions can also be detected using the internal PMTs setting the pinhole at wide open (detection width for “green” fluorescence 500-550nm, detection width for “red” fluorescence 565-605nm). Comparison of fluorescence intensities in images acquired with descanned and non-descanned geometries with the same laser settings.

(c) Acquisition of 3D z-stacks using z-compensation

In order to acquire a Z-stack, the laser settings need to be increased as the depth increases. The acquisition of a z-stack can be done either manually or automatically using the "z-compensation" tool. Automatic "Z-compensation" in combination with detection using the NDD detector allows adjusting the 2-photon laser power ("gain") and the detector “smart” gain (linear compensation by AOTF and PMT). Manual z-compensation is also an option, where separate z-stacks with different laser and detection settings can be acquired and be combined in the end using the LAS AF software.

(d) Spectral unmixing of the dyes

In case of simultaneous detection, **spectral unmixing** might be needed. Spectral unmixing can be performed using the spectral dye separation tool of the LAS AF software.

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(e) Laser lesion

A 2-photon laser lesion can also be induced followed by time-lapse monitoring of EGFP microglial cells, which immediately respond and migrate to the lesion site in order to repair the damage. To perform laser-lesion a field of interest is selected and the region to be photobleached is placed in the center of the image. In the xyzt mode, z-stacks are acquired for 10 minutes before the bleaching step. Each z-stack should be acquired in 1 minute. Bleaching is performed by zooming in the region of interest, tuning the laser at 800nm (wavelength at which maximum power is achieved), and increasing the laser settings "trans" and "gain" to almost 100% (duration 1-2 frames). After photobleaching, the 2-photon laser is tuned at the initial wavelength and zoom and the other settings are set to the initial pre-bleaching values. A time-lapse sequence is acquired for 20-60 minutes after the bleaching. Each z-stack should be acquired in 1 minute.

(f) Image analysis

Image processing and analysis involve creation of 3D reconstructions, rotated movies, time-lapse series and analysis of the movement of the microglial cells using LAS AF and Imaris software.

Acqkowledgements:

We would like to thank Dr Era Taoufik for performing the intravenous dye injections.

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