RIIP

Reseau International Institut Pasteur

International Course 2016

"Cell Biology and Infection: Digital Image Processing/Analysis Tools for Quantitative Light Microscopy Imaging"

Hosting organization:

Hellenic Pasteur Institute

Light Microscopy Unit

Sponsors:

RIIP, Institut Pasteur, Institut Carnot Pasteur MI

Hellenic Pasteur Institute

Main Organizer:

Haralabia Boleti

Co-organizers:

Jean Christophe Olivo-Marin,

Evangelia Xingi & Fabrice de Chaumont

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Preface

Advanced Light Microscopy is an essential tool for research and diagnosis in most areas of biomedical sciences. Direct visualization of live biological samples when combined with quantitative analysis of data extracted from digital images, can provide novel insight or reinterpretation of biological questions investigated in the past only by biochemical methods.
The latest developments in Fluorescence microscopy (i.e. confocal and multiphoton microscopy, fluorescence time lapse video microscopy, techniques as FRAP, FRET, FLIM, FCS for molecular imaging and determination of cell dynamics, super resolution microscopy etc) that allow molecular 3D and 4D imaging of multiple fluorochromes simultaneously and the determination of cellular dynamics at 5D, make an absolute requirement the use of highly sophisticated computer software for 3D modeling and quantitative analysis and interpretation of the imaging data**.**
The rapid progress in Live Cell Imaging, Bioinformatics and Digital Image analysis tools has created a gap in the training of scientists using advanced imaging approaches in Digital image processing. A real need therefore exists for continuous training of the microscopy users on the various image analysis software. This training will enable them to extract quantitative information from still images or videos from live imaging experiments and thus, fully exploit the information contained in their microscopy data to draw accurate and in depth conclusions about the biological phenomena they study.
This course is addressed to scientists from all Institutes of the Institut Pasteur International Network (IPIN) but also to scientists from all academic institutions. We hope that it will serve as an "incubator" for new networking and new possibilities for collaborations.
Finally, we are very happy to emphasize the real **international character of this course, where** students and instructors from **11 different countries and 4 continents** participate.

Course description

This **5-day practical course** entitled "Cell Biology and Infection: Digital Image Processing/Analysis Tools for Quantitative Light Microscopy Imaging**"** focuses on topics related to biomedical applications in the fields of infectiology and cell biology.

Its main objective is to provide **theoretical and practical training** in basic and advanced concepts and methods of digital image analysis and in the use of two open source (Icy and Fiji) software for quantitative assessment of fluorescence microscopy data through:

- Lectures
- Hands-on practical training with participants' microscopy data or data provided by the instructors
- Informal discussions and tutorials with experts in the field

The course is addressed to PhD students, post doctoral fellows and young investigators who carry out research projects requiring fluorescence microscopy imaging and digital image processing.

Hellenic Pasteur Institute

http://www.pasteur.gr/?lang=en

The **Hellenic Pasteur Institute (HPI**), a member of the Institut Pasteur International Network, is a non-profit research foundation that operates through a bilateral agreement between the Greek Government and the Institut Pasteur in Paris, France. Since its establishment in 1920, the HPI remains faithful to its **mission**, which is the prevention and treatment of Diseases through Basic **Research**, **Education** and **Public Health Services**.
Education at HPI focuses on the training of young scientists (PhD, MSc and diploma students) and the dissemination of specialized scientific information to specific public target audiences. Besides the training of young researchers in the HPI laboratories, HPI researchers organize national and international scientific training courses for researchers and graduate students on cutting-edge technologies. Additionally, in the context of Science and Society activities, the HPI has organized courses on biosciences and bioethics for secondary school teachers and hosts secondary- and primary-school students' visits for educational purposes. **The Hellenic Pasteur Institute Light Microscopy Unit** (HPI-LMU) (http://www.pasteur.gr/?page_id=2444&lang=en), is one of the most modern and well-equipped imaging facilities in Greece. It provides advanced light microscopy tools and services to in-house and visiting scientists and is involved in several training projects at national and international level.

RIIP (Reseau International Institut Pasteur)

IPIN (Institut Pasteur International Network)

http://www.pasteur.fr/en/international

The International Network of Pasteur Institutes and Associated Institutes (Reseau International des Instituts Pasteur et Instituts Associés) comprises 33 Institutes in all continents which have signed in 2010 a new Collaboration Agreement of the Institut Pasteur International Network. The Pasteur Institute in Paris has been the core for the development of this International Network.

For over a century, the contribution of the Institut Pasteur International Network essentially coincides with the history of discoveries that has marked the development of the biomedical sciences and the struggle against some of humanity's greatest health problems: The boubonic plague by Yersin, typhoid fever by Laveran, diphtheria by Roux and Ramon, tuberculosis by Calmette and Guerrin. Also, this contribution includes some of the early steps of molecular biology by A. Lwoff, J. Monod and F. Jacob and the isolation of the AIDS virus by J. L. Montagnier. Eight Nobel prizes, eight landmarks in the history of medicine.

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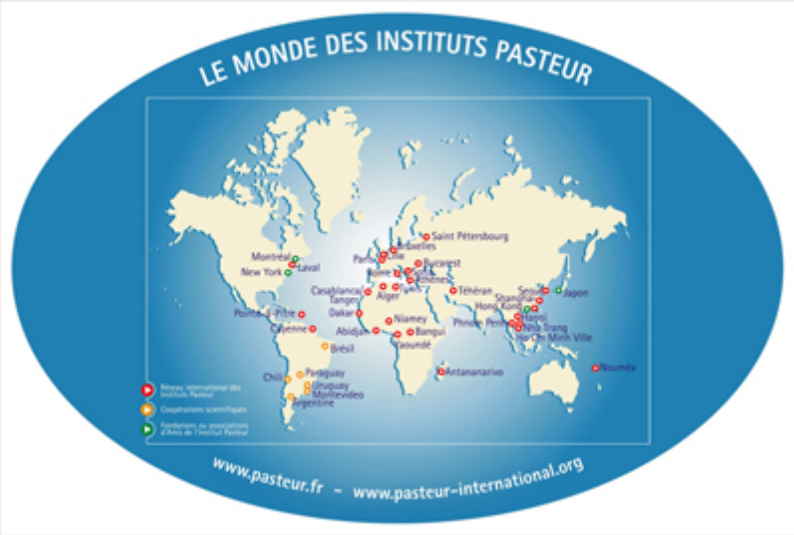
Every year, the Institut Pasteur International Network organizes and finances teaching & training within the Network reserved for the Institutes' researchers, the technicians or the students and for the exterior personnel (ministries, universities), who can then use their experience in the other national or regional structures.



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Institut Pasteur Institut Pasteur

http://www.pasteur.fr/en

Since its creation in 1887, the Institut Pasteur has become famous throughout the world as a symbol of science and French culture. For 120 years, it has been contributing to the prevention and treatment of infectious diseases through research, teaching and public health initiatives.

Across the globe, thousands of researchers, trained or working at the Institut Pasteur, share values that form the very core of the Pasteurian community: an original scientific approach, an ongoing preoccupation with applying research to public health needs, professional ethics, a desire to care for people regardless of their background or nationality, and an outward-looking attitude, sharing knowledge and expertise with the international community. These guiding principles make the Institut Pasteur a unique institution which values exchanges and partnerships.
With its unique setup and prestigious history, the Institut Pasteur has always stood at the forefront of

innovation, adapting to the rapidly developing world of biological research and its applications, in particular in the field of biotechnology, but most importantly to the fields of infectious diseases,

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microbiology, virology and immunology.

Carnot Foundation Maladies infectieuses

http://www.instituts-carnot.eu/en/carnot-institute/pasteur-mi

The Carnot Institute "Pasteur Infectious Diseases" consists of five thematic departments of the Institut Pasteur (virology, microbiology, parasitology and mycology, infection and epidemiology, cell biology and infection), which in association with the CNRS and INSERM is dedicated to research on infectious diseases. It brings together rising and completed research activities, and makes them subject of many research partnerships with SMEs and large companies in the pharmaceutical and diagnostics. Pasteur MI strives to combine a unique know-how with multidisciplinary scientific approaches to offer its industrial partners relevant answers to major economic and public health issues related to

- Mechanisms of bacterial, viral, fungal and parasitic pathogenesis
- Genetics and predisposition to infections
- Microbiota and human health
- Integrative biology
- Clinical and epidemiological studies
- New antimicrobial strategies
- Biotechnologies

"Cell Biology and Infection: Digital image processing/analysis tools for Quantitative Light Microscopy Imaging"

Program

1st Day, Monday, July 4

Processing and quantification of biological images/ tools and applications

08:30-09:00	*Registration*
09:00-09:30	**Haralabia Boleti**, *Hellenic Pasteur Institute* Introduction to the course/Opening remarks
09:30-10:30	**Participants** Introductory presentations on their research projects and aims related to the course
10:30 - 10:50	*Coffee break*
10:50 - 11:40	**Jean-Christophe Olivo- Marin**, *Institut Pasteur* Image analysis and computer vision tools for the processing and quantification of biological images. Icy, the open source computer software for bioimaging
11:40 - 12:30	**Szymon Stoma**, *ScopeM, ETH Zurich,* Introduction to image processing and analysis using FIJI/ImageJ

Practical session on ImageJ/Fiji

12:30 - 13:30	ImageJ/Fiji hands on practical Instructors: **S. Stoma, E. Xingi, F. de Chaumont**
13:30 - 14:30	*Lunch*
14:30 -16:30	ImageJ/Fiji hands on practical
16:30 - 16:50	*Coffee break*
16:50 - 18:30	ImageJ/Fiji hands on practical
	Free time
21:00-23:00	*Welcoming Dinner (students and instructors)*

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2nd Day, Tuesday, July 5

Processing and quantification of microscopy data / tools and applications

09:00 - 9:45	**Dimitra Thomaidou**, *Hellenic Pasteur Institute* Analysis of digital images and videos derived from multiphoton microscopy intravital imaging
09:45 - 10:30	**Fabrice de Chaumont**, *Institut Pasteur* Icy: new features and future
10:30 - 10:50	*Coffee break*

Practical session on Icy

11:00- 13:30	Icy hands on practical *Instructors*: **F. de Chaumont, A. Dufour, S. Dallongeville, L. Danglot, J. Mutterer**
13:30- 14:30	*Lunch*
14:30 -16:30	Icy hands on practical
16:30-16:50	*Coffee break*
16:50-18:30	Icy hands on practical

3rd Day, Wednesday, July 6

Cell motility and dynamics of phagocytosis and intracellular traffic

09:00- 09:45	**Alexandre Dufour,** *Institut Pasteur* Deciphering cellular morpho-dynamics using Bioimage Informatics
09:45-10:30	**Chiara Zurzolo**, *Institut Pasteur* Imaging Prions spreading in neurodegenerative diseases: Role of Tunneling Nanotubes (TNTs)
10:30 - 10:50	*Coffee break*
10:50 - 11:35	**Florence Niedergang**, *Institut Cochin* Imaging phagosome formation, closure and maturation in normal and virus-infected macrophages.

Sponsored by:

Practical session on ImageJ/Fiji

11:40- 13:30	ImageJ/Fiji hands on practical *Instructors*: **S. Stoma, E. Xingi, F. Nierdengang, J. Mutterer**
13:30-14:30	*Lunch*
14:30 - 16:30	ImageJ/Fiji hands on practical
16:30-16:50	*Coffee break*
16:50-18:30	ImageJ/Fiji hands on practical

4th Day, Thursday, July 7

Super-resolution and high throughput Imaging based screening

09:00- 09:45	**Lyndia Danglot,** *Institut Jacques Monod* Morphometric analysis of neuronal networks: from memory to super-resolution structure of the synapse
09:45-10:30	**Franc Perez,** *Institut Curie* Systematic analysis of the secretory pathway in mammalian cells
10:30 - 10:50	*Coffee break*
10:50 - 11:35	**J. Pizarro Cerda,** *Institut Pasteur* Automated High Throughput Microscopy-based RNAi screening of mammalian cell invasion by the bacterial pathogen *Listeria monocytogenes*

Practical session on Icy

11:40- 13:30	Icy hands on practical *Instructors* : **F. de Chaumont, A. Dufour, S. Dallongeville, F. Perez, J. Mutterer**
13:30- 14:30	*Lunch*
14:30 -16:30	Icy hands on practical
16:30-16:50	*Coffee break*
16:50-18:30	Icy hands on practical

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5th Day, Friday, July 8

Imaging of pathogen host interaction

09:00- 09:45	**Freddy Frischknecht**, *Center for Infectious Diseases, Heidelberg University* Understanding malaria parasite migration
09:45-10:30	**Isabelle Tardieux**, *Institut of Advanced BioSciences, Univ. Grenoble Alpes* Multi-imaging approaches to decode how *Toxoplasma* parasites invade target cells
10:30 - 10:50	*Coffee break*
10:50 - 11:35	**Jerome Mutterer,** *Institut de Biologie Moléculaire des Plantes* Image ethics for publication (what can be done and what should be avoided)

Informal tutorials with the instructors/ Practice on Icy and ImageJ/Fiji

11:40- 13:30	*Instructors* : **F. de Chaumont, A. Dufour, S. Dallongeville, S. Stoma, J. Pizarro Cerda, E. Xingi, I, Tardieux, F. Frishchnecht, J. Mutterer** Participants will practice with the help of the instructors on their projects using tools from Icy or ImageJ/Fiji
13:30- 14:30	*Lunch*
14:30 -16:00	Preparation of participants' presentations
16:00-17:45	Participants' presentations on the results from their course projects
17:45-18:00	**Haralabia Boleti,** *Hellenic Pasteur Institute* Closing remarks, *END of Course*
19:30- 21:00	***Cultural / Social event*** *Guided tour to the New Acropolis Museum*
21:00-23:00	*Farewell Dinner*

ABSTRACTS

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OUTLINES

of

PRACTICAL TRAINING

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Image analysis and computer vision tools for the processing and quantification of biological images.

Icy, the open source computer software for bioimaging

Jean Christophe Olivo Marin

Bioimage Analysis Unit, Institut Pasteur, Paris, FRANCE

An increasing number of biological projects aim at elucidating the links between biological function and phenotype through imaging and modelling the spatiot emporal characteristics of cellular or organism dynamics. In a firts part, we will present and discuss some recent developments of robust and automated tools and software for flexible and robust quantitative assessment of 2D/3D+t dynamic imaging data from biological projects. Thanks to these tools, it is possible in a large number of experiments to automate the extraction of quantitative data from images and to facilitate the understanding of the biological information contained therein.

Then we will present Icy, a free open-source software that provides an integrated web-based development platform for bioimage informatics applications. Icy offers a common platform for both image analysis scientists developing new algorithms and biologists seeking for a powerful and intuitive tool for image analysis applications. It combines indeed a community website for contributing and sharing tools and material, and software with a high-end visual programming framework for seamless development of sophisticated imaging work flows. Icy features more than 150 applicative plugins covering such diverse tasks as image enhancement, filtering, active contours, cell segmentation and tracking, particle detection and tracking. Icy is available at http://icy.bioimageanalysis.org/.

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Introduction to image processing and analysis using FIJI/ImageJ

Szymon Stoma

ETH (Swiss Federal Institute of Technology), Zürich, Switzerland

Handling of microscopy images in scientific research requires at least basic understanding of digital images. A digital image translates to numbers and is hence a quantitative by nature. During the introductory lecture I will discuss the very basics of the numerical nature of digital images including image representation, spatial and bit-depth resolution, image dimensions, lookup tables, basic mathematical operations and convolution-based filters. During the presentation I will introduce ImageJ, a public domain software package for image processing and analysis developed initially by Wayne Rasband (http://imagej.net). Many scientists especially biologists use ImageJ for their research, so the package became a *de facto* a standard tool for dealing with images in Biology.

Analysis of digital images and videos derived from multiphoton microscopy intravital imaging of the neurovascular niche following neuroinflammation

Dimitra Thomaidou

Laboratory of Cellular and Molecular Neurobiology, Hellenic Pasteur Institute, Athens, Greece

The importance of the Neurovascular Niche (NVU) in the maintenance of cerebral homeostasis is being gradually established during the last years. The term "Neurovascular Unit" (NVU) is used to describe the integrated system of vascular and neuronal cells and the surrounding glia working in concert to enable proper brain homeostasis and function. The NVU is affected in many CNS conditions and plays a role in their pathology. Neuroinflammation is a common feature of many neurodegenerative and neuroimmunological diseases and if uncontrolled, it can result in the constant activation of microglia and astrocytes, two of the major components of the NVU. This can generate a neuroinflammatory environment which can further promote neurodegeneration. Thus, the study of the dynamic cellular interactions in the pathological NVU could contribute to the identification of numerous potential targets for treatment in the context of many CNS pathologies. The dynamics of such processes can be adequately captured by intravital in vivo imaging, which allows the study of cellular responses to environmental stimuli and cell-cell interactions in the living brain in real time. Accordingly, in the present study 2-Photon Laser Scanning Microscopy (2P-LSM) has been applied to follow in real time the morphological changes and interactions of microglia, astroglia and the brain vasculature under neuroinflammatory conditions induced by the lipopolysaccharide (LPS), which is a potent activator of systemic inflammation. Analysis of collected imaging data, following intravital brain imaging using Imaris v.7.4.2 and Icy Bioimage Analysis Software, revealed cellular changes both in astroglia and microglia upon LPS exposure, characteristic of a strong and long-term neuroinflammatory response accompanied by quick astroglia and microglia activation. Additionally, the observed infiltration of inflammatory cells from the periphery inside the brain tissue is considered to be indicative of a decrease in the blood-brain-barrier (BBB) permeability during these conditions. Further study will focus on the establishment of BBB leakage in our model and the possible protective role of the polarized astrocytes under these circumstances.

Icy: new features and future!

Fabrice de Chaumont

Bioimage Analysis Unit, Institut Pasteur, Paris, FRANCE

Icy is a free open-source bio-image analysis software. During its 4 years of life it has been continuously improved and continuously brings new features to users and developers. Today Icy is used by more than one thousands of regular users which appreciate its intuitive GUI, its ray-traced 3D visualization and its cutting-edge analysis methods. Users can also adapt and create new algorithms with script and the graphical programming protocol designer and store them on the Icy website which centralizes all resources (http://icy.bioimageanalysis.org). Thus it makes available plug-ins, protocols and scripts to everybody. Centralization also allows searching directly from within the application for specific features and enables it in a one-click install. During the last months, Icy has received new exciting features: revisited 3D rendering based on latest VTK version and a new powerful Undo framework. Icy is now addressing big data: we introduced streaming capability, cluster deployment and headless calls. Finally, Icy is always evolving, thanks to the feedback of all its users on http://icy.bioimageanalysis.org/support!

Deciphering Cellular Morphodynamics using Bioimage Informatics

Alexandre Dufour

Bioimage Analysis Unit, Institut Pasteur, Paris, FRANCE

Cell deformation and migration are key factors involved in numerous aspects of cell development, immune responses, cancer and infectious diseases. Our team develops image analysis tools and software to extract quantitative information from multi-dimensional, multi-modal live microscopy, with the aim to derive mathematical models of cellular morpho-dynamics. Throughout this talk, I shall describe some of our algorithmic developments to a) automatically track multiple cells in 2D/3D microscopy in cluttered environments and low signal-to-noise conditions, b) efficiently describe the 3D morphology of cells despite their natural shape variability, and c) extract biophysical quantities at the intra-cellular level directly from imaging data. Finally, we shall emphasize the need for open-source tools and software by presenting the Icy platform (http://icy.bioimageanalysis.org), a next-generation software dedicated to the bioimaging communities by providing the latest developments in image acquisition, visualisation, analysis and computational modeling.

Imaging Prions spreading in neurodegenerative diseases: Role of Tunneling Nanotubes (TNTs)

Chiara Zurzolo

Membrane Traffic and Pathogenesis Unit, Pasteur Institute, Paris France

Neurodegenerative diseases (NDs) such as Prion disease, Alzheimer's disease (AD), Parkinson's disease (PD) and Huntington's disease (HD) are part of a larger group of protein misfolding disorders (PMDs) characterized by the progressive accumulation and spreading of protein aggregates of different sizes - oligomers, protofibrils or fibrils-, which ultimately can assemble into extracellular amyloid deposits and/or intracellular inclusions. The best-characterized example of PMDs is prion diseases, which are caused by the conversion of the normal form of the prion protein (PrPC), to a misfolded form (PrPSc) through "template conformation changes". Like in Prion diseases, misfolded forms of ASYN, tau, Abeta and Htt proteins associated with AD, PD and HD can be transmitted experimentally in cellular and in animal models where it can act as 'seeds' to recruit the endogenous protein into aggregates (seeding process). However, the mechanism of intercellular transfer is still obscure.

We have recently described a novel mechanism of PrPSc transmission through Tunneling Nanotubes (TNTs). TNTs are actin-based fine protrusions connecting sparse cells in culture and represents a novel mechanism of cell-to-cell communication. By using live and quantitative imaging we have analysed TNT formation and their role in prion transfer. We found that TNT are mediating both exogenous and endogenous PrPSc transfer between infected and naïve mouse neuronal cells and between bone-marrow dendritic cells and primary neurons. Furthermore, mutant polyQ Htt aggregates appear to highjack TNTs as well as fibrillar and oligomeric ASYN assemblies. We propose that TNTs might contribute to the progression of the pathology of neurodegenerative diseases associated with the spreading in the brain of misfolded protein assemblies. I will discuss our novel data mainly based on imaging techniques on the mechanism of TNT formation and their role in the spreading of protein aggregates involved in neurodegenerative diseases.

Imaging phagosome formation, closure and maturation in normal and virus-infected macrophages

Florence Niedergang

Institut Cochin (Inserm U1016, CNRS UMR 8104, Université Paris Descartes, Sorbonne Paris Cité, Paris, France

Phagocytosis and degradation of microorganisms or debris by macrophages is crucial for pathogen clearance and resolution of inflammation[1]. The internalization step of phagocytosis relies on a profound remodeling of the plasma membrane and the cytoskeleton. The local, intense and transient actin polymerization is the force that deforms the plasma membrane to form pseudopodia[1,2].

We set up an original experimental approach, based on Total Internal Reflection Fluorescence (TIRF) Microscopy to monitor the spatio-temporal reorganization of membrane and actin in nascent phagosomes in three dimensions in living macrophages[3]. We observed that vesicular delivery occurs at the base of the phagocytic cup where actin is depolymerized, while F-actin is present in extending pseudopods. We showed that the NF-kB-signaling protein B cell lymphoma/leukemia-10 (Bcl10) is unexpectedly involved in actin remodeling during phagocytosis by controlling the traffic of intracellular vesicles bearing the AP1 adaptors and the OCRL phosphatase[4]. Using this system, we also revealed that a crosstalk between actin and dynamin-2 takes place during phagosome formation, and that dynamin-2 plays a critical role in the effective scission of phagosomes from the plasma membrane[5].

Macrophages are a main target and reservoir for the Human Immunodeficiency Virus (HIV)-1. HIV-infected macrophages exhibit defective functions that contribute to the development of opportunistic diseases. We demonstrated that macrophages infected with HIV-1 exhibit defects in phagocytosis, which are due to the major virulence factor Nef, perturbing focal exocytosis of intracellular compartments[6]. More recently, we identified the viral factor Vpr as a modulator of the microtubule dependent endocytic trafficking that alters localization of microtubule binding proteins EB1 and $p150^{Glued}$, leading to strong alterations in phagosome movement and phagolysosome biogenesis[7]. To measure the speed of centripetal movement of phagosomes in individual HIV-GFP-infected macrophages, we used a combination of bright field and fluorescence confocal microscopy[8]. We also used correlative electron microscopy to analyze the development of bacteria in HIV-infected macrophages.

Together, our data aim to understand how opportunistic pathogens develop in HIV-1 infected macrophages.

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Morphometric analysis of neuronal networks: from memory to super-resolution structure of the synapse

Lydia Danglot

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Keywords: synapse, plasticity, super-resolution microscopy, SIM, STORM, brain tile imaging.

The nervous system contains billions of neurons that communicate with each others via specialized junctions: the synapses. Synaptic dysfunction is at the heart of the etiology of a wide range of neurological and psychiatric disorders (Alzheimer's disease, autism spectrum disorders or schizophrenia). Indeed, many diseases are characterized by altered distribution and / or mis-apposition of the pre and post-synaptic sites. We propose here to describe imaging techniques and image analysis tools to detect, count and characterize such defects through spatial statistics. We will describe imaging techniques used to study from neuronal network, to single molecule localization microscopy. These wide technical approaches will be described through the characterization of our biological models in the lab which is the characterization of transgenic mice line knocked out for a vesicular SNARE protein involved in fusion process.

Indeed, the establishment, maintenance and plasticity of the synapse require the transport, recycling and degradation of membrane-associated synaptic molecules via membrane trafficking mechanisms. Vesicular and target-SNAREs play a central role in all membrane trafficking pathways. Neurons express several v-SNAREs including TI-VAMP/VAMP7 which is involved in Golgi to plasma membrane transport particularly of membrane micro domains, pre synaptic exocytosis, lysosomal secretion and autophagosome biogenesis and secretion. We previously found that TI-VAMP/VAMP7 KO mice are viable and characterized by decreased brain weight, increased ventricle volume as measured by MRI, and increased anxiety suggesting a role for VAMP7 in higher brain functions [1]. In addition, VAMP7 was previously found to be particularly enriched in the hippocampus, a brain structure involved in learning and memory, its expression being both pre- and post-synaptic.

Here we found that VAMP7 KO mice showed increased memory performance and a decreased memory decline in old animals compared to wild-type. We are now deciphering at the tissular, cellular and molecular level what could explain such increased performances.

We investigated the neuronal morphology in situ by tile imaging of the entire brain (Golgistaining) to realized mosaic image at a good resolution in order to discriminate dendritic arbors. Those results indicated profuse dendritic pyramidal arborization in specific regions of the KO brain suggesting altered morphological and potentially functional post-synaptic properties. This was further confirmed by electron microscopy which unraveled both pre and post-synaptic structural modifications in the KO. Neuronal network and spine density is actually approached by tile confocal imaging and 3D reconstruction.

At the synapse level, we found altered synaptic biochemical content in glutamatergic receptors and associated molecules by biochemical approaches. Determining the precise localization of molecules using image based analysis is a standard and powerful tool to probe molecular assembly in cellular compartments. We further investigated the localization of the synaptic molecule by multicolor super-resolution microscopy (SIM and STORM). Principle and imaging technics will be presented. We are

currently developing image analysis software in collaboration with T. Lagache and JC. Olivo Marin team (Institut Pasteur) to analyze precise distance between coupled synaptic molecules by robust statistical approaches [2]. While standard colocalization techniques have been widely used in standard fluorescence microscopy, the emergence of super resolution microscopy made obsolete the classical overlap approaches and calls for new methods of analysis. We developed a method and software (SODA: Statistical Object Distance Analysis) that statistically quantifies the relative spatial positioning of several molecules' populations. The method computes the coupling distance and specifically quantifies the morphology (size, shape, intensity) of coupled spots. We will present the example of the apposition of three consensual synaptic molecules in primary hippocampal neurons with structured-illumination microscopy and STORM microscopy. We will present advantage of both methods and explain why they are complementary.

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Systematic analysis of the secretory pathway in mammalian cells

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It is now clear that multiple routes exist in cells to transport proteins and lipids toward various compartments. To analyze these routes, there is a strong need for a versatile, highly synchronized assay that can be used at physiological temperature, in living cells. We developed the RUSH system (Retention Using Selective Hooks), a two-state assay based on the reversible interaction of a Reporter protein with a Hook protein stably localized in a donor compartment. This system can be used to study and quantify the trafficking of very diverse proteins of different size, shape and destined to various compartments. The RUSH system can be used for real time monitoring of transport in living cells as well as for High Throughput screening using automated imaging.

We are using the RUSH assay at multiple scales to monitor the transport of proteins in cells and analyze their intracellular signatures, to map the diversity of trafficking routes in terms of biochemical composition, to integrate these routes with other cellular machineries, like cellular adhesion and polarization, and to screen diverse chemical libraries and identify potential therapeutic molecules.

During this lecture, I will present data obtained using spinning disk microscopy, TIRF microscopy and High Content imaging. Data obtained using traction force microscopy and cells grown on patterns will also be shown. Analysis of these data was performed using FIJI/ImageJ and the In Cell Analyzer software.

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Automated High Throughput Microscopy-based RNAi screening of mammalian cell invasion by the bacterial pathogen *Listeria monocytogenes*

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The gram-positive bacterium *Listeria monocytogenes* is the causative agent of human listeriosis, a food-borne disease characterized by gastroenteritis, meningitis and abortions. A crucial stage in infection is the bacterial internalization and intracellular replication process in phagocytic and normally non-phagocytic cells. Indeed, *Listeria* is able to promote its entry within mammalian cells, to disrupt its internalization vacuole, to proliferate in the host cell cytoplasm and to use an actin-based motility system to move within cells and to spread to neighboring cells (Pizarro-Cerda et al. 2012). We have recently designed high-throughput microscopy-based screens in order to: a) investigate late cellular infection, and b) discriminate between bacterial entry events and escape from the internalization vacuole.

To investigate late cellular infection, we established an assay relying on the cytosolic detection of InlC, a bacterial protein from the internalin family that is highly expressed and secreted by intracellular *Listeria*. By using antibodies to label InlC, we are able to differentiate infected from non-infected cells (Kühbacher et al. 2013). We used therefore this microscopic assay to investigate cellular pathways required for *Listeria* infection using an experiment protocol that includes: **a)** the reverse RNAi transfection of HeLa cells in 384-well plates for 72 hours using diverse human RNAi libraries, **b)** the infection of transfected cellular monolayers with a *L. monocytogenes* EGDe.PrfA* strain expressing the enhanced-GFP for 1 hour, **c)** the killing of non-invading bacteria with a gentamicin solution for 4 hours, **d)** the fixation/permeabilization of cells and **e)** their labeling with DAPI for nuclear staining, with fluorescent phalloidin for actin staining and for InlC using polyclonal anti-InlC antibodies to specifically identify *L. monocytogenes* infected cells. Using the public software CellProfiler (http://cellprofiler.org/) (Broad Institute), we then: **I)** used a 'Mixture of Gaussian' (MoG) thresholding method to segment the nuclear DAPI staining and identify the number of cells per image, **II)** used a 'Propagation/Otsu Global' thresholding method to identify the cytoplasm of cells using the actin staining, and **III)** classified 'infected' versus 'non-infected' cells using the signal from the InlC labeling. Infection indexes were estimated by dividing the number of infected cells by the total number of cells within a specific monolayer for a specific RNAi treatment, and a Z-score is calculated after logarithmic transformation of the infection indexes -which permits the comparison among results from plates generated in different experiments (Rämö et al. 2014). Using this approach, we identified the

existence of different Arp2/3 complexes in cells which differentially regulate actin polymerization at *Listeria* entry sites and during cytoplasmic actin-based motility (Kühbacher et al. 2015).

To discriminate between bacterial entry events and escape from the internalization vacuole, we generated a *Listeria* strain which expresses in its surface a β-lactamase, which allows cleavage of the fluorescent probe CCF4. In cells loaded in their cytoplasm with CCF4, bacteria which can not escape their phagosome can not have access to the probe, which upon excitation will emits a FRET fluorescent signal at 520 nm. However, when bacteria are able to escape their phagosomes, their surface β-lactamase will cleave the CCF4 which will inhibit FRET and display fluorescence at 447 nm. Using an Opera Imaging System coupled with the Acapella software (PerkinElmer) we first analyzed the FRET signal to identify cells in which *Listeria* vacuolar escape took place. In the same samples, we then performed a differential bacterial staining (Kühbacher et al. 2014) to differentiate extracellular from total bacterial populations, and again using the Opera Imaging System/Acapella software we estimated in the proportion of bacteria which invaded cells (Quereda et al. 2016), discriminating entry from vacuolar escape.

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Understanding malaria parasite migration

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Malaria parasites are transmitted by mosquitoes, which inject the parasites into the skin of the host. There, parasites migrate at extremely high speeds penetrating the tissue and searching for a blood vessel that they need to enter to continue their journey. We aim at understanding the rapid motility. Like eukaryotic motility it depends on an interaction of actin and myosin as well as substrate contact. However, unlike most eukaryotic motility malaria parasites do not change their shape during migration. I will present our mixed approach of generating parasite lines expressing fluorescent or altered actin binding proteins and how we investigate these lines using a range of biophysical assays and micro-structured or nano-patterned substrates.

These assays include the use of (i) soft substrates with embedded fluorescent microspheres to investigate traction forces generated by the parasites (Munter 2009); (ii) the use of poly-dimethylsiloxan pillar arrays as structured micro-environments to investigate migration patterns of parasites (Hellmann 2011); (iii) the use of optical tweezers to investigate the membrane flow of parasites as well as force generation on the parasite surface (Quadt 2016); the use of tiny gold beads for generation of nano-structured surfaces to investigate the numbers of surface proteins needed for substrate adhesion and their influence on motlity (Perschmann 2011). We utilize standard image analysis routines based on ImageJ as well as custom written plugins.

During the afternoon practical sessions, I will be discussing some examples of bad image arrangements for figure generation in the hope to stimulate students to only generate perfect figures in their theses and papers.

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Multi-Imaging Approaches to Decode How *Toxoplasma* Parasites Invade Target Cells

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Apicomplexa constitute a large phylum of protozoan parasites, which includes medically important human pathogens such as *Toxoplasma* and *Plasmodium*, the agents of toxoplasmosis and malaria respectively. Occurring within the context of immunocompromised human beings, the public health threats imposed by *Toxoplasma* are directly associated with the ability of the transient extracellular *Toxoplasma* crescent shaped stage, called tachyzoites (7 to 3 µm length/width), to **invade** and expand its population size, inside most non phagocytic and phagocytic cells. Host **cell invasion, a unique and spectacularly rapid process** (lasting few seconds) is arguably one of the most scrutinized scientific questions in Apicomplexan biology with the relevant perspective of designing anti-invasive molecules to prevent disease. A shared multi-step model of host cell invasion by Apicomplexan parasites has been proposed about 30 years ago, with the parasite establishing a tight junction with the host cell plasma membrane and then **actively penetrating** into a nascent parasitophorous vacuole. Of note, recent datasets generated with both genetically engineered *Plasmodium* and *Toxoplasma* parasites and host cells have cast doubt on and revealed flaws in this model, **the major highly debated questions** being:

1- the force contribution of each partner during invasion.

2- the role of the host cell cortex to control junction shaping and function during parasite entry.

We will discuss imaging approaches we have undertaken to address these issues that include:

- Fluorescent Speckle Microscopy to follow actin flow and turnover in live cells following microinjection of fluorescent actin. It relies on the use of conventional wide-field fluorescence light microscopy, on digital imaging with a low-noise CDD camera and on Matlab image acquisition and analysis.

- Fluorescent confocal real time video-microscopy of invasion events with multi-channel image acquisition using a spinning disk and a coolsnap HQ2 camera driven by the Metamorph software. Image processing includes trajectory analysis and allows **kinematic modeling** (Matlab) to monitor *Toxoplasma* pre-invasive and invasive behavior. These approaches have also allowed monitoring simultaneously host cell actin and membrane dynamics during parasite invasion.

- Automatic image capture and data analysis for quantitative *Toxoplasma* invasion assays performed on 96 well plates and simultaneously under various settings using the Olympus Scan^R

automated inverted microscope and Scan^R software (object and sub-object detection, parameter calculation, specific gating and classification schemes).

- Force measurement using **Atomic Force Microscopy coupled to fluorescent imaging** to attempt getting direct insights on the force contribution of tachyzoites during attachment/ invasion of host cells and on the changes of host cell cortex tension during these events.

These imaging techniques have been proved decisive to properly analyze *Toxoplasma* phenotypes for wild type and "invasion-related" mutants. These data also provide new tools and concepts to study membrane and cortex tension of mammalian cells, a topic of increased relevance to understand cell motility, division and features of post mitotic cells.

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Image ethics for publication

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Microscopy images carry a considerable amount of data. The lectures and practical trainings provided in this course aim at presenting state of the art methods to obtain usable images and extract significant data from them. Another step forward is publishing those images. The publication step is of major importance as the published data is expected to guarantee both a perennial link to the original raw data and sufficient information for research reproducibility. I'll discuss common pitfalls associated with publishing image data and present existing tools that can be used by scientists. On one side I will present solutions for mandatory storage of original raw data, that range from laboratory or institution secured storage to scientific online data stores, in a context where the amount of acquired raw data itself becomes challenging. On the other side, I'll suggest and demonstrate the use of available tools for securing reproducible image processing workflows, as available in different image acquisition or image analysis software.

ImageJ/Fiji hands on practical session

Szymon Stoma

ETH, Zürich, Switzerland

The goal of these workshop sessions is to introduce the most important concepts of working with images in biology, while teaching you how to use some very popular image analysis software. To achieve this goal I will go over various usages of FIJI/ImageJ. Learning to use FIJI/ImageJ properly often enables people to understand quantitative aspects of image processing and analysis. We start with GUI usage, and then extend this to writing ImageJ macros*. Images are everywhere in science, so if you continue in this area you are quite likely to encounter them at some point - perhaps directly through your own need to perform analysis, in helping colleagues, or in evaluating whether some analysis reported in a paper has been done sensibly.

Part 1 Understanding images and FIJI/ImageJ basics
Part 2 Handling dimensions (2D+ images) & measurements
Part 3 Pixel processing & thresholds
Part 4 Segmentation, filters & transforms
Part 5 Towards quantitative image analysis
(**Part 6** Automating analysis)*

Monday 4 July: Part 1-3

- Image examination (bit depths, saturation, offset, dynamic range, LUT)
- Comparison of different image formats/ 8-bit conversion
- Color images
- Image formats
- Image restoration
- Measurements
    - Intensity measurements in multiple regions
    - Biological interpretation of intensity measurements
- Thresholding and particle analysis
- Multidimensional images

Wednesday 6 July: Part 4-6*

- Mathematical operations
- Image filtering
- Smoothing to detect objects in noisy images
- Segmentation
- Background subtraction on an image
- (Macros)*
    - Reproducibility
    - Automation
    - Workflow optimization

*) depending on group tempo, interactions with ICY, etc.

Icy hands on practical session

Fabrice de Chaumont, Stéphane Dallongeville, Alexandre Dufour
Bioimage Analysis Unit, Institut Pasteur

We present Icy, an open source and free software dedicated to the bio-image analysis. Icy provides an integrated platform that aims at bridging the gap between developers and users, by combining: a) an open-source image analysis software, offering a powerful and flexible environment for developers such as applied mathematicians to write algorithms fast and efficiently; b) a common set of tools to view and manipulate data, and a set of plugins to perform specific quantification or analysis on images; c) a community-based website centralizing all plugins and resources to facilitate their management and maximize their visibility towards users.

During the course, we will focus on: a) using the software for analysis and data visualization purposes; b) understand the file format, effect of compression over images on the noise data, and how much noise data is important in the image analysis; c) the use of look up table and false color representation; d) multiple use of ROI and their edition; e) creating ROI automatically through segmentation plugins; f) detecting spots and performing tracking analysis; g) using batch processing.

Then we will focus on the autonomy Icy provides to the biologists by using its graphical programming and scripting capabilities through various examples.

We will then finish by demonstrating how to publish original work created during this workshop.

All along the course, participants will be invited to use their own images, and apply the "Icy" methods directly on their analysis.

Tuesday 5 July

Icy free software http://icy.bioimageanalysis.org/

- Installing the software
- Exploring the image with a 2D visualization
- Using the look up table, and understanding the dynamic of the image
- Explain image acquisition to avoid the loss of information
- Understanding the noise in an image, and why its evaluation is needed
- Using the Region of Interest
- Using a ray-tracer in 3D to visualize the images.
- Practical example: an analysis of an atomic force microscopy acquisition
- Browsing for functionalities and Installing a plugin
- Detecting spots in 2D/3D or 4D

- From the quantitative result to biological deduction: good practice and traps
- Performing a tracking and analyzing the results
- Tracking of big objects with deformable models
- Track objects over time in 2D and 3D
- Generate object statistics on length, speed, duration and distance from origin, displacement straightness etc.

Thursday 7 July

- General introduction to co-localization analysis
- Correlation and Overlap Based methods
- Pearson and Mander's Coefficient
- Statistical significance and quantification
- Object-based methods
- Molecule detection and spatial analysis
- Statistical significance and quantification
- Co localization Studio and Co localization Simulator
- Impact of noise on the different methods
- Test with synthetic images
- Perspectives: 3D, time and non-parametric methods
- Creating complex analysis workflow and batch processing with graphical programming and scripting

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and

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