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NOVEL TECHNOLOGIES FOR STUDIES OF STRUCTURAL AND FUNCTIONAL CONNECTIONS

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NOVEL TECHNOLOGIES FOR STUDIES OF STRUCTURAL AND FUNCTIONAL CONNECTIONS

THESIS FOR DOCTORAL DEGREE (Ph.D.)

By

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Thursday 20th of December 2018 at 9:30

ABSTRACT

To understand the mechanisms underlying the correct functioning of an organ it is important to study its architecture and how the interactions between cells are leading to a specific function. Specifically, the connections that form in the brain are related to the pattern of activation that neurons have, and can help to understand what is the function of each region. Combining the structural knowledge with functional studies is crucial to understand how the cells communicate and propagate the depolarization. Another way to understand the mechanisms underlying tissue functionality is to try to replicate its features and inspect if the resulting behavior is similar to the original one.

In this thesis I am describing different tools that we developed to inspect the cells connectivity from the architectural and functional point of view, focusing on imaging, analytic and engineering techniques.

To inspect the connection within the brain, in paper I we developed a microscopy system capable of performing fast volumetric imaging of large cleared samples (called LSTM, Light Sheet Theta Microscopy). LSTM is built upon the LSM (Light Sheet Microscopy) system, but instead of illuminating the sample from the sides –which leads to a physical constrain to the samples lateral dimension or depth– the light sheet is scanned on the imaging plane from an angle smaller than 90°. Therefore this approach eliminates the constraints on the lateral size without compromising the image quality and speed. Furthermore, it overcomes the LSM limitation that leads to huge scattering on the center part of the sample. In fact, LSTM images each plane with the same intensity leading to homogeneous x-y acquisition throughout the whole dept. This system can help to create maps of long ranging connections of neurons of intact rodents organs (eg brain) and can theoretically be used to acquire un entire human brain, slab by slab, in a reasonable amount of time.

In paper II we propose a tool to inspect the evolution of living cultures for an extended period of time. To do so, we developed a mini-microscope to be placed in the incubator that performs long lasting recordings and automatically detects the Regions Of Interest (ROI), calculates the intensity profiles, and compresses the data after every time-point. This system (called XDscope) is designed to limit the user interaction with the culture, minimize the light exposure and to ease the process of getting the desired information out of the experiment and store as little data as possible. Using the XDscope we performed long term monitoring of GCaMP6 expressing neurosphere (NSP) networks for over 2 weeks, showing that the cells behavior is not affected by the long acquisition. Furthermore we used the system to evaluate the uptake mechanism of p-HTMI, an LCO (Luminescent Conjugated Oligothiophene) over the NSP network, showing that the targeted cells are progenitor cells as expected, since the fluorescent cells are mainly located around the spheres. Finally we investigated further the specific target of p-HTMI within the cells performing double labeling with proteins that seemed to be in the targeted area. From the results it seems like GM130/Golga2, a protein

that facilitates the transportation between ER and Golgi apparatus has a high percentage of overlap with the molecule.

Finally in paper III we tried to mimic the features and the cell spatial arrangement of a leaving tissue to infer similar properties to an engineered construct. We propose an innovative strategy to integrate a patterned gold microelectrode into a flexible biomimetic hybrid actuator with double muscle-like patterned layers made using PEG (Polyethylene Glycol) and CNT-GelMA (Carbon Nano Tubes- Gelatin Methacryloyl). The CNT-GelMA patterned layer acted as a substrate for cell culture to induce maturation of cardiac muscle cells, while the PEG layer acts as the backbone of the whole membrane. The resulting muscle-like biohybrid actuator showed excellent mechanical integrity with an inserted Au microelectrode and advanced electrophysiological functions with strong muscle contractions. Therefore, we successfully fabricated a biomimetic hybrid actuator with muscle-like pattern, and controllable movement under an electrical field produced by integrated electrodes.

LIST OF SCIENTIFIC PAPERS

I. ***Light sheet theta microscopy for rapid high-resolution imaging of large biological samples***
Bianca Migliori*, Malika S. Datta*, Christophe Dupre, Mehmet C. Apak, Shoh Asano, Ruixuan Gao, Edward S. Boyden, Ola Hermanson, Rafael Yuste, and Raju Tomer
BMC Biology (2018) 16:57.

II. ***XDscope: A new tool for live cell fluorescent imaging***
Bianca Migliori, Ola Hermanson , and Raju Tomer
Manuscript (2018)

III. ***Electrically Driven Microengineered Bioinspired Soft Robots***
Su Ryon Shin*, **Bianca Migliori***, Beatrice Miccoli, Yi-Chen Li, Pooria Mostafalu, Jungmok Seo, Serena Mandla, Alessandro Enrico, Silvia Antona, Ram Sabarish, Ting Zheng, Lorenzo Pirrami, Kaizhen Zhang, Yu Shrike Zhang, Kai-tak Wan, Danilo Demarchi, Mehmet R. Dokmeci, and Ali Khademhosseini
Advanced Matererials (2018), 1704189

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LIST OF ABBREVIATIONS

AFM	Atomic Force Microscopy
BO	Brain Organoid
CCD	Charge-Coupled Device
CNT	Carbon Nano Tubes
COLM	Clarity Optimized Lishtsheet Microscopy
diSPIM	dual view iSPIM
ECM	Extra Cellular Matrix
EGF	Epidermal Growth Factor
EEG	Electroencephalogram
ER	Endoplasmic Reticulum
ETL	Electrically Tunable Lens
ExM	Expansion Microscopy
FGF	Fibroblast Growth Factor
fMRI	functional Magnetic Resonance Imaging
FWHM	Full Width at Half Maximum
GCaMP	Genetically Encoded Calcium Indicator (GECI)
GelMA	Gelatin Metacryloyl
GSC	Glioblastoma-derived Stem cell-like Cells
GUI	Graphical User Interface
iSPIM	inverted Selective Plane Illumination Microscopy (iSPIM),
LCO	Luminescent Conjugated Oligothiophene
LCP	Luminescent Conjugated Polythiophene
LED	Light Emitting Diode
LSM	Light Sheet Microscopy
LSCM	Line Scanning Confocal Microscopy
LSTM	Light Sheet Theta Microscopy
MIPL	Maximum Illumination Path Length
MRI	Magnetic Resonance Imaging

NA	Numerical Aperture
NIRS	Near Infra Red Spectroscopy
NSP	NeuroSPheres
OCPI	Objective-Coupled Planar Illumination
OPM	Oblique Plane Microscopy
PDMS	Poly(DiMethylSiloxane)
PEG	PolyEthylen Glycole
p-HTMI	Penta-hydrogen thiophene methyl imidazole
PDI	Protein Disulphide Isomerase
PSF	Point Spread Function
sCMOS	scalable Complementary Metal-Oxide Semiconductor
SCAPE	Swept Confocally Aligned Planar Excitation
SEM	Scanning Electron Microscope
TAG	Tunable Acoustic Gradient
TDI	Tensor Diffusion Imaging
Thy1-YFP	Thymus cell antigen 1- Yellow Fluorescent Protein
TIF	Tif (Tagged Image Format)
TMSPMA	3-(TriMethoxySily) Propyl MethAcrylate
WD	Working Distance

1 INTRODUCTION

1.1 Imaging and labeling

Biomedical imaging is a series of techniques used to capture the morphology and the functionality of a biological structure or phenomena.

Imaging and labeling techniques are now fundamental and irreplaceable tools in most phases of scientific research. These tools form an essential part of the process of understanding biological systems by providing morphological, structural, metabolic and functional information. Microscopy is not anymore limited to capturing images of a sectioned and sliced fixed sample, but ranges from live imaging to 3D mapping of entire cleared organs, from in vivo recording of neural activity to mapping location and identity of specific molecules.

As imaging techniques were advancing, the neuroscience field often pioneered new visualization tools. In this chapter there is a description of the evolution of the most used visualization techniques from the first neuronal layers drawings to the latest advances of the field.

1.1.1 History of imaging: from bright field to fluorescence

One of the first scientists that started to address the challenge of representing the fine details of neuron's morphology was the Nobel Prize Ramón y Cajal in the beginning of the nineteenth century. He drew a detailed representations of neurons by observing the cells and drawing the structures and interconnections of the dendritic tree for the first time(DeFelipe 2015) using a rudimentary microscope with natural light as illumination source.

After the introduction of artificial light and cameras into the microscopes, enormous progresses were made in the field.

The first instruments that started to be very diffused were very simple bright field microscopes with transmitted light. The samples were usually illuminated from the bottom and imaged from the top. This type of imaging does not provide a good contrast, so it was hard to observe the finest detailed using this methodology. To improve the image contrast two major approaches were taken: improve imaging systems and label the specific object of interest. Following this direction the new field of Fluorescence imaging started to rise (Zanacchi, Bianchini et al. 2014).

Fluorescence imaging is the visualization of fluorescent dyes or proteins as labels for molecular processes or structures. It enables a wide range of experimental observations including the location and dynamics of gene expression, protein expression and molecular interactions in cells and tissues.

To achieve the visualization of fluorescent labels, many different microscopy systems have been used, each one with different characteristics and advantages.

To generate a fluorescent image, the specimen is illuminated with a light source of a specific wavelength. This illumination causes the excitation of a fluorophore that in response emits a photon at a longer wavelength. The emitted photon is then separated from the illumination light by means of a selective filter, and captured on a photosensitive sensor or through the eyepieces (Lichtman and Conchello 2005) (**Fig. 1**). A multicolor fluorescence image is the result of a combination of several single-color images. The most widespread fluorescent microscope is called epi-fluorescence microscope. Johan S. Ploem, who introduced the principle of using incident light in contrast to transmission microscopy, was its inventor(Ploem 1999). One big advantage of using this technique for fluorescence microscopy is to avoid the detection of emission light delivered by the light source.

Typically, an epi-fluorescent microscope is composed by a light source to excite the fluorophore, an excitation filter to modulate the light's wavelength, a dichroic mirror to selectively reflect the excitation light frequencies and refract the emitted fluorophores, and an emission filter to selectively get the emitted light (**Fig. 1**). The limitation of this system is that the samples are evenly illuminated by the light source. This means that all the fluorophores hit by the light are excited at the same time, including the out of focus planes. This leads to low quality, as in the same image both the in-focus and the out of focus planes are captures, and to the impossibility of optically section the sample, since it can't selectively image one plane at a time.

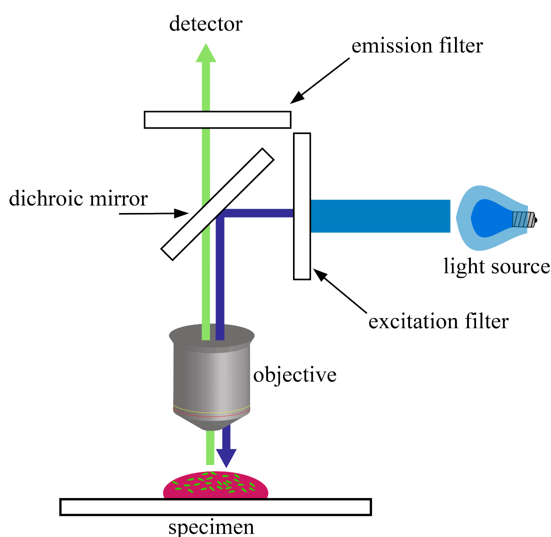

Figure 1 Schematic representation of fluorescence microscopes

To overcome these limitation, in 1957 Marvin Minsky invented a new system called Confocal Scanning Microscope (Minsky 1988).

The confocal microscope introduces 2 main elements: a point illumination and a pinhole in front of the detector (**Fig.2a**).

The point illumination is generated by an aperture that makes the illumination beam smaller, and the pinhole (placed in an optically conjugated plane) eliminates the out of focus signal coming from the out of focus planes. Since just the signal generated by the fluorophores very close to the focal plane can be captured, the resulting image has an increased optical resolution compared to the conventional wide-field fluorescence microscopes.

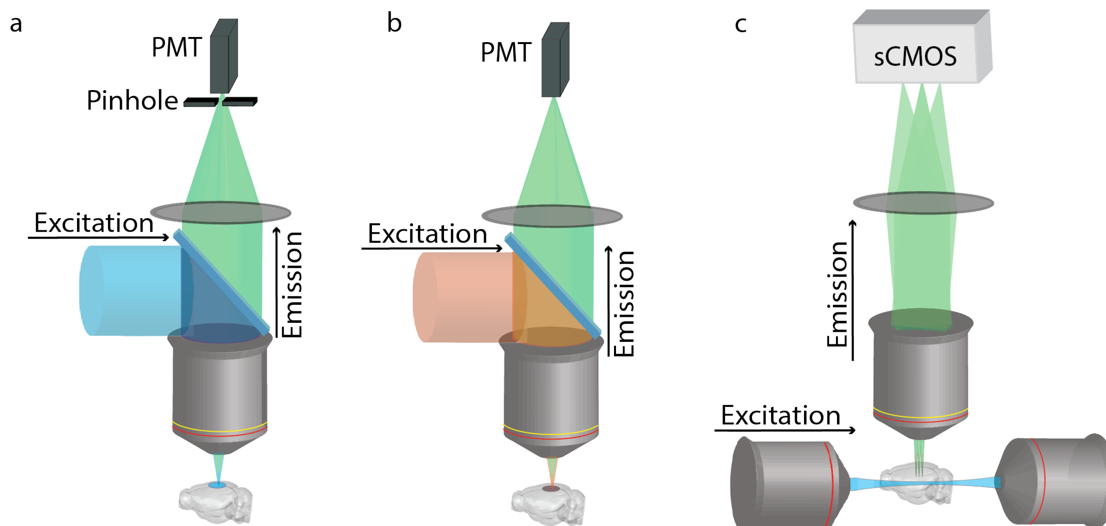

Figure 2 Schematic illustration of the working mechanism of a) confocal microscopy, b) Two photon microscopy and c) Light Sheet Microscopy

This revolutionary new approach enabled for the first time optical sectioning in thick (tens of micrometers) fluorescently labeled samples, thereby allowing 3D reconstruction without the need for ultrathin physical sectioning (Conchello and Lichtman 2005).

Nevertheless, as much of the signal coming from sample fluorescence is blocked by the pinhole, with the increased resolution comes also a decrease of signal intensity, which leads to longer exposure times (Carter 1999).

To address this limitation, the camera is replaced with a sensitive detector, (usually a PhotoMultiplier Tube (PMT)), which transforms the light signal into an electrical one that is recorded by a computer (Rai and Dey 2011) (**Fig. 2a**). An advantage of this signal transformation is that the images are detected by a computer, which can divide frequencies that are closer to each other making it possible to use more labeling colors.

Another way to achieve optical sectioning is to use the Two-Photon excitation-adsorption principle, concept first described by Maria Goeppert-Mayer (Göppert-Mayer 1931) in her doctoral dissertation in 1931.

This principle states that when two photons hit at the same time a molecule they combine their energies to promote the molecule to an excited state, which then results in the emission of a photon along the normal fluorescence-emission pathway (Denk, Piston et al. 1995, Helmchen F. 2006) (**Fig. 2b**).

A Two-photon microscope illuminates the molecule with 2 consecutive photons at approximately half the energy load necessary to excite the molecule. The resulting emission of the fluorescent photon is typically at a higher energy then either of the excitatory photons.

The lower energy excitation laser travels at a longer wavelength compared to the one-photon excitation. The advantage of using longer wavelengths is that they scatter less than the shorter

ones, which leads to improved resolution in the z direction and imaging penetration (Kaminer, Nemirovsky et al. 2013).

Finally, two photon microscopy increases the accessible imaging depth (to hundreds of micrometers) even in living tissue samples, which are less damaged compared to one photon excitation, and adaptive-optics approaches have improved imaging depth further (Tang, Germain et al. 2012).

Nevertheless, fluorescence light microscopy still is not able to perform volumetric acquisition of intact opaque tissues (Tomer, Ye et al. 2014). A common work-around to this limitation has been to slice the biological tissue into thin sections, and then acquire every portion of it using a confocal or a two-photon imaging system (Micheva, Busse et al. 2010, Ragan, Kadiri et al. 2012). This approach requires a long time, both for slicing and acquisition, and does not preserve the tissue from damages and losses.

1.1.2 Volumetric imaging and light sheets

To get the best result in terms of acquisition time and quality the ideal approach would be to image the intact and labeled tissue as a whole. To achieve this goal the first thing to do would be to reduce the opacity of the tissue making it transparent by chemically reducing the scattering of light traveling through the tissue sample. Recent advances in tissue clearing techniques are granting new access to large intact biological tissues.

New methods have emerged showing highly effective results, like Scale (Hama, Kurokawa et al. 2011), CLARITY (Tomer, Ye et al. 2014), SeeDB (Ke, Fujimoto et al. 2013), CUBIC (Susaki, Tainaka et al. 2015), iDISCO (Renier, Wu et al.), and uDISCO (Richardson and Lichtman 2015). These techniques typically involve a cocktail of chemicals to dissolve and remove membrane lipids (the main cause of light scattering) and an optical smoothening step (by incubating in a specific refractive index RI-matching liquid).

For example, tissue clearing with CLARITY starts with the perfusion of a tissue sample using a solution of acrylamide, bisacrylamide, formaldehyde, and a thermal polymerization initiator. This is followed by a thermally initiated polymerization reaction, which results in a highly cross-linked meshwork of hydrogel fibers and amine-containing biomolecules. Lipid membranes are then removed either by passive thermal clearing in a buffered SDS solution at 37°C (Tomer, Ye et al. 2014) or by an active electrophoretic process (Chung, Wallace et al. 2013)—the result is an intact, highly transparent tissue-hydrogel hybrid.

Combining these clearing methods with innovative genetics and viral vectors-based labeling approaches is allowing the access to the molecular structure and the functional architecture of intact tissues.

Once the sample is cleared and labeled, still there is the need of an imaging system capable of acquiring full volumes without having to section the tissue into thin slices. Light-sheet microscopy (LSM) techniques are considered to be suitable means to keep up with scientific advances in tissue clearing (Migliori 2016) (**Fig. 2c**).

The concept of LSM was introduced more than a century ago by Siedentopf and Zsigmondy (Siedentopf and Zsigmondy 1902), who created the ultramicroscope, which collected sunlight using a lens that focused on a horizontal bilateral slit to reduce the vertical dimension (**Fig. 3**).

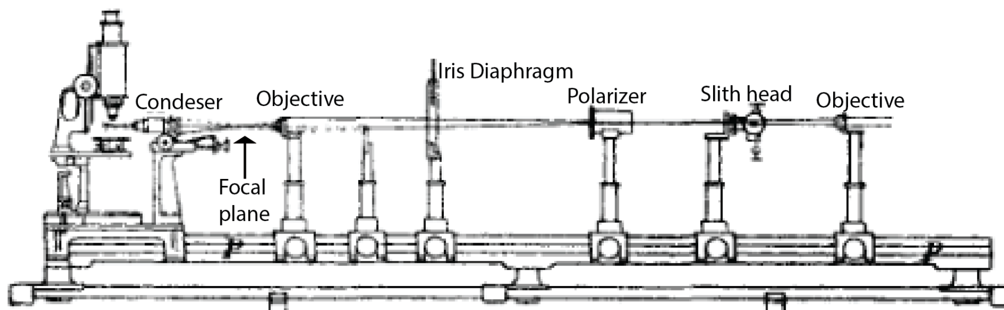

Figure 3: Overview of the assembly of the ultramiscope, the first prototype of LSM

An optional Polarizer allows for polarization, while the Iris Diaphragm ensures removal of side-reflected light from the Slit Head. A chisel-shaped Iris Diaphragm is used to adjust the dimension of horizontally focused light, which is passed through a lens and a condenser to generate a thin sheet of light. An orthogonally arranged in-focus wide-field detector allows visualization of the illuminated plane.

It is only during the past two decades though that LSM techniques have emerged as highly effective methods for rapid volumetric imaging of both fixed and small living samples, thanks to parallel advances in lasers, detectors, computer hardware and software, and genetic labeling methods.

LSM microscopy involves orthogonal illumination of the sample's transversal plane with a thin sheet of light, and detection of emitted signal with an orthogonally arranged wide-field imaging system (**Fig. 2c**). The optical sectioning is achieved by the confinement of illumination to the imaging focal plane of interest. This configuration provides two main advantages: Minimal energy load, resulting in low photobleaching and phototoxicity; and high imaging speeds (LSM images are more than two orders of magnitude faster than other high-resolution microscopy techniques, such as confocal and two-photon microscopy), due to the simultaneous detection of the entire illuminated optical plane by fast sCMOS or CCD cameras.

These low-energy-load and high-speed capabilities make LSM ideally suited not only for volumetric acquisition of entire fixed organs, but also for long-term imaging of live samples (Huisken, Swoger et al. 2004). Indeed, LSM has been successfully used to capture the embryonic developmental dynamics of small transparent model organisms such as

zebrafish (Keller, Schmidt et al. 2008), *C. elegans* (Keller, Schmidt et al. 2008), and *Drosophila* larvae (Tomer, Khairy et al. 2012), for capturing the high-resolution details of subcellular processes (Huisken and Stainier 2009), and for brain-wide mapping of neuronal activities in small model organisms.

Once this technique started to become established in the scientific community, many researchers started to upgrade and customize their systems accordingly to their research needs.

COLM (CLARITY-optimized light-sheet microscopy) (Tomer, Ye et al. 2014) for instance was developed to be optimized for high-quality imaging across entire intact clarified samples.

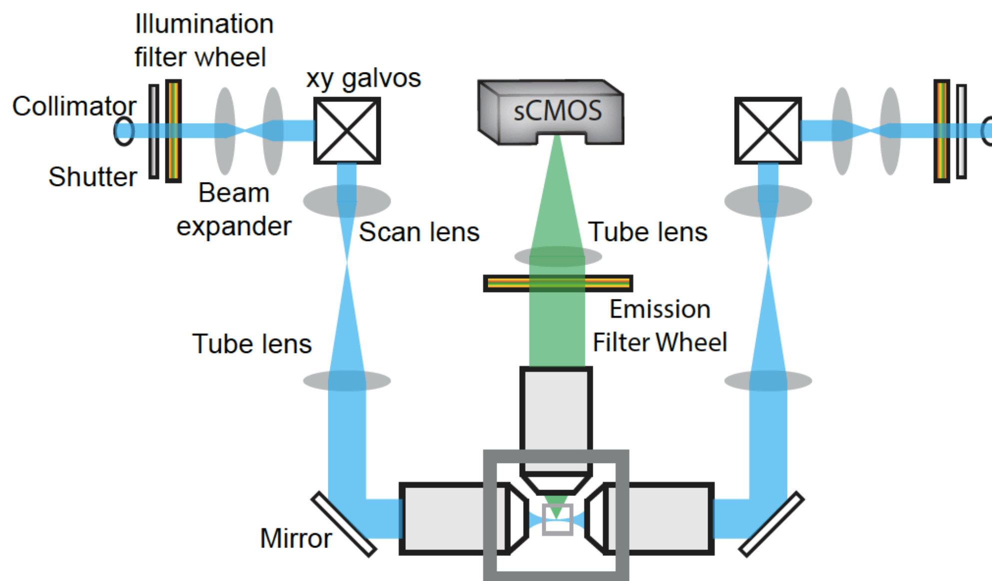

Figure 4: Schematic of COLM system(Migliori 2016)

The COLM technique enabled rapid, high-resolution imaging of entire, intact mouse brains and nervous systems(Migliori 2016) (**Fig. 4**).

The main innovations introduced by the COLM systems are first an optically homogeneous sample-mounting framework that minimizes optical aberrations, especially across the sample depth. A clarified intact organ is mounted in a cuvette (made of specific transparent material with matching RI to minimize the interfaces), attached to a xyz stage by a set of adapters inside the sample chamber, which is filled with a matching RI liquid. This mounting strategy mitigates the effect of changes in RIs in the detection paths, and also allows the use of appropriate immersion objectives for detection (e.g., CLARITY-optimized long working-distance objectives).

Second, the implementation of a synchronized detection-illumination procedure leads to higher imaging quality and depth by reducing the out-of-focus background. This was performed by synchronizing the line-by-line detection of sCMOS camera chips with fast, pencil-beam scanning of the sample to generate a virtual light sheet (virtual slit effect, Tomer et al 2014).

Third, the introduction of an automatic adaptive parameter-correction procedure to adjust for misalignments (of the light-sheet and detection focal plane). The method involves quick calibration to automatically estimate the alignment parameters at defined imaging positions distributed sparsely across the sample. Linear interpolation of the alignment parameters in all three dimensions facilitates high-quality imaging throughout the sample. The result is one of the first demonstrations of an automatically self-correcting adaptive LSM.

A limitation of standard LSMs is that the stage motion limits volumetric imaging speed, since the sample or the entire microscope assembly has to be moved to acquire the entire specimen (which also leads to vibrations that can affect the acquisition). This limitation makes it hard to acquire in a 3D fashion fast live events like calcium signaling.

SPED (spherical aberrations-assisted extended depth-of-field) (Tomer, Lovett-Barron et al. 2015) system was developed to address these limitations(Migliori 2016) (**Fig. 5**).

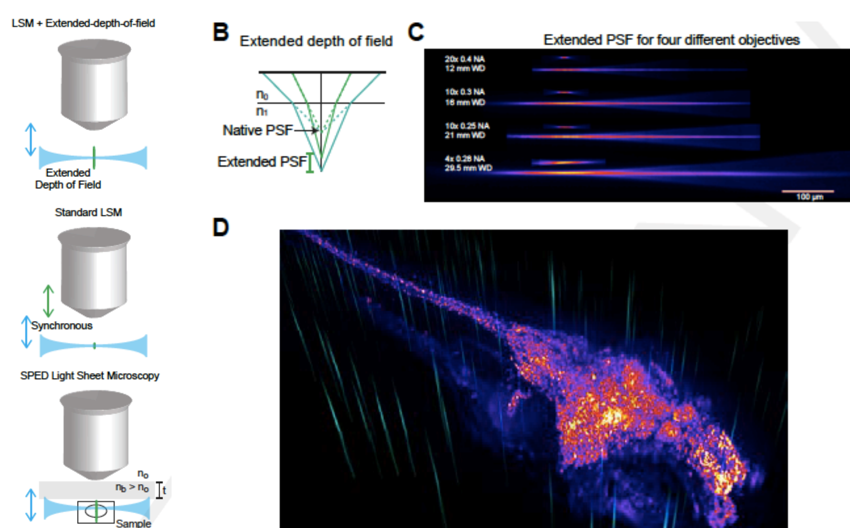

Figure 5 a) SPED combines the optical sectioning of light-sheet microscopy with extended-depth-of-field. (**b**) A ray-tracing example demonstrates the use of a RI change to elongate the PSF. (**c**) SPED-elongated PSF of four different objectives covering a range of specifications: For each objective, the top is native PSF measured in air, and the bottom is elongated PSF. (**d**) Volume rendering from a high-speed live volumetric imaging dataset of a zebrafish larva expressing calcium indicator GCaMP, which acts as a reporter for neuronal activity.

The basic concept is that the final optical point spread function (PSF) in LSM is the result of the intersection of the light sheet and the detection objective PSF. The lateral resolution is thus determined by the detection objective's numerical aperture (NA), whereas the axial resolution derives from the thickness of the illumination light sheet. Therefore, by axially elongating the detection PSF while keeping the lateral extent constant, it becomes possible to acquire an entire 3D volume at very high speed and resolution by rapidly scanning the light sheet alone, and bypassing the need for a piezo motor to move the sample or the objective. This method though requires post processing and deconvolution to achieve the final image.

Another way of upgrading the speed of volumetric acquisition is to add an Electrically Tunable Lens (ETL) to the detection arm (Fahrbach, Voigt et al. 2013). ETLs are polymeric lenses made by a thin membrane as an interface between a liquid viscous mean and air.

Once a voltage is applied, the polymer deforms changing the focus of the waves that passes through them. This electric driven deformation happens very quickly, making it possible to use them to rapidly shift the focus on the sample instead of physically move the stages.
At this point, LSMs various systems have already enabled such unique experimentation capabilities as the capture of cellular dynamics in developing embryos; mapping of the structure of entire intact, chemically cleared organs; and revelation of functional dynamics in the brains and nervous systems of small, transparent model organisms.

1.1.3 Novel labeling techniques

Fluorescent tagging or labeling is a way to detect and identify biomolecules within a biological tissue. As human knowledge about physiology and anatomy was rising, there has been an increasing need of identifying the finest structures and components of biological tissues. Following this need, fluorescence labeling has emerged to improve the study of molecular structure and interactions at cellular and subcellular level.

The basic concept of this technique is to attach a fluorescent tag to a molecule that then binds to a specific target within the tissue. Different strategies of molecular labeling have been developed, including genetic, enzymatic, chemical, protein labeling, and more recently colorimetric biosensors, electrochemical sensors, photochromic compounds and biomaterials.

Immunohistochemistry, which is the most diffused labeling technique, involves the use of specific antibodies that binds selectively to the target protein. To achieve the labeling, there are two different approaches: a direct and an indirect approach (**Fig. 6**).

Figure 6 Schematic of direct and indirect detection methods.

In the direct approach fluorochrome-labeled secondary antibodies are used for fluorescent labeling of the primary antibodies, which are targeting the molecule of interest. The indirect method uses non-labeled antibodies for the particular biomolecules as the primary antibodies. This technique usually requires permeabilization of the plasma membrane and a fixation procedure, which makes it unsuitable for live-imaging.

Chemical fluorescent labeling can visualize cellular structures such as nucleus, plasma membrane, and cytoskeletons with chemical reagents coupled with fluorochromes that bind to the biomolecules specific to particular structures. Chemical fluorescent probes are easy to use and are best suited for counter-staining. Cell permeable fluorescent dyes are also useful for live-cell imaging.

To capture the activation dynamics and the functionality of live cells and tissues, there are many techniques that utilize calcium indicators. Fluorescent probes that exhibit a spectral response upon Ca2+ binding have enabled to directly visualize changes of intracellular Ca2+ concentrations in living cells by fluorescence microscopy (Partridge 2015) (**Fig. 7**). Most of calcium indicators are derivatives of the Ca2+ chelators, but lately genetically encoded calcium indicators are spreading among the scientific community. The most used one is GCaMP, which is created from a fusion of green fluorescent protein (GFP), calmodulin, and M13, a peptide sequence from myosin light chain kinase. The advantage of this technique is that it can be genetically specified for studies in living organisms.

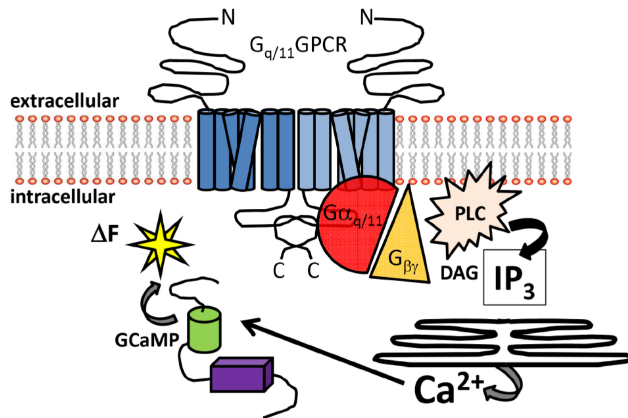

Figure 7: Schematic of the working mechanism of the Calcium indicator called GCaMP. (Partridge 2015)

With the advances of the imaging fields, new approaches are rising to follow the increasingly specific needs of every application. A novel approach that involves the use of small probes specific for a distinct biomolecule or structural element have proven useful for recording biological events. In this regard, luminescent conjugated oligo- and polythiophenes (LCOs and LCPs) have been utilized as target-specific probes that change their emission depending on the structural motif of a distinct target molecule even in a complex environment such as tissue (Sigurdson, Nilsson et al. 2007, Sigurdson, Nilsson et al. 2009, Berg, Nilsson et al. 2010). LCOs and LCPs contain a repetitive flexible thiophene backbone, and a conformational restriction of the thiophene rings upon interaction with a biomolecule leads to a distinct optical fingerprint from the dyes. These new type of molecules can be manipulated and redesigned to express fluorescence in different wavelengths, and to target different biomolecules. Furthermore, they don’t need long time for reaction nor elaborated protocols, but they just accumulate in the target area in a fast a passive way.

1.2 Study of cell networks: connection, function and stimulus propagation

The brain is a very complex organ composed of mainly two types of cells: neurons and glial cells. The neurons are cells that are able to respond to physical and chemical stimuli by modifying the ions concentration of their membrane. They can produce and propagate a signal in form of variation of electrical potential, and transmit it to another cell using specific junctions called synapses. The glial cells’ function is mainly to sustain the neurons by providing mechanical stability and selecting the molecules that from the blood go in the neuron and vice versa. Furthermore they regulate the synaptic communication and the

neuronal growth. To understand the mechanisms underling the brain functionality it is important to see how the cells are organized, how the neurons are interconnecting and how the signal is propagated between cells.

1.2.1 Neuron connectivity in the brain

In the human brain there are around 100 billion neurons and around one trillion of interconnections. Every neuron innervates a target and is innervated by other cells. Since every neuron can receive thousands of nerve endings, it takes and processes the signals coming from different neurons, creating a very intricate network. The receiving signals can be excitatory or inhibitory, and the neuron gets excited or inhibited accordingly to the sum of all the stimuli that is receiving in a given moment. Consequentially, each neuron can transmit only one type of information (its own) that is influenced by the activity of the neurons that are connected with it. The neurons that are part of the same network are distributed and connected accordingly to a specific predefined genetic sequence and is associated with a specific function (**Fig. 7**). Hence, the presence in a circuit of inhibitory neurons modulates the final output of the network, witch results or not in a specific action.

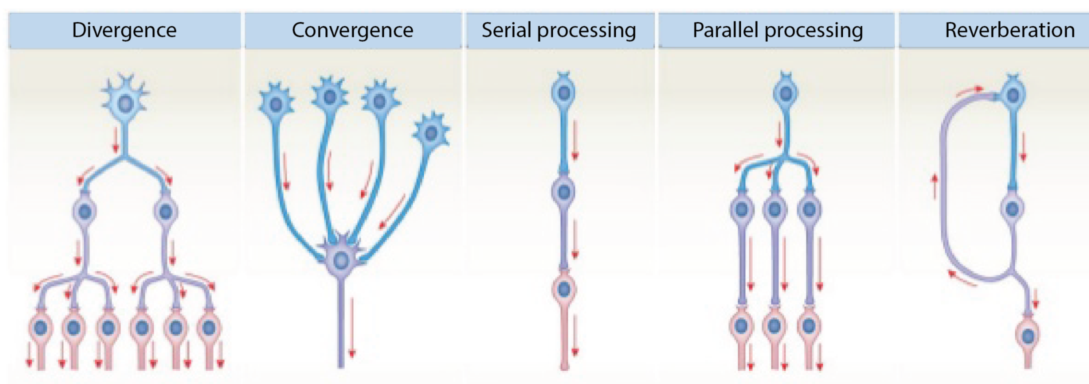

Figure 7 Examples of neural circuits and pulse propagation

To advance our understanding of the brain, an entire field is dedicated to inspect the structural shape of neural networks and highlight the connections among increasing hierarchical levels (macro-, micro- and nano-connectomes) (Crosson, Ford et al. 2010).

Macroconnectomics is focused on identifying the connections between regions. In a mammalian brain there are around 500 to 1000 regions and around 100 trillion connections between them. One of the main imaging techniques used to study connectomes at macro level is fMRI (functional Magnetic Resonance Imaging) and its variations.

MRI techniques build an image by detecting the radio frequency signal that is emitted by a hydrogen atom after being excited by an oscillating magnetic field(Buxton 2012). The shape of the different tissues is defined by the rate in which the hydrogen goes back to the relaxed state, giving a direct measure of the amount of water present in the specific tissue (hence the density). In fMRI the aim is to detect the change of blood flux (BOLD (Blood signal))

(Singleton 2009) associated with the increase of neuronal activity. Since blood is a paramagnetic material, it tends to concentrate the magnetic flux, resulting in a reduction of the MRI signal.

Other approaches are to associate the activation of brain regions to other bio-signals, such as electrical signal (EEG, Electroencephalogram), temperature differences (TDI, Tensor Diffusion Imaging), Near Infrared Adsorption Spectrum (NIRS). Most of these techniques are used in combination with a pre acquisition at higher resolution so that the fast and less detailed signals coming from the activation are overlapped to a reference structural image.

Thanks to these approaches there is now an accepted map of the brain where we know where specific actions come from (eg. motion, sensation, vision, ecc..)

Microconnectomes are considered the connections between specific neurons at cellular level. The connections are mainly studied using optical imaging tools, commonly fluorescent microscopy. Nowadays it is still very hard to map the entire nervous system of mammals, so either scientists focused on finding out the connections at cell level of small specific circuits, or they tried to map the entire wiring in small organisms (eg C. Elegans or Drosophila) (Shoter et all). C. Elegans is one of the few organisms where the nervous system was mapped entirely. Caenorhabditis Elegans is a 1mm long completely transparent nematode. This feature allows labeling and imaging of its cells without having significant optical issues related to penetration. C. Elegans have a very simple nervous system. Out of 959 somatic cells, 302 are neurons with a very simple structure and stereotypical morphology and can be divided into 3 types: motor, sensory and interneurons. The motor neurons were identified from the neuromuscular junctions; specific morphological and location features characterize the sensory neurons, and the remaining cells were classified as interneurons(White, Southgate et al. 1986). The wiring between neurons is mainly arranged to minimize economy and the connectivity follows the small world organization, meaning that not all the neurons are connected directly, but every one of them can reach any other with a small number of steps.

The Drosophila (Drosophila melanogaster) is a little fly, and its brain has around 100,000 neurons, hence it is much bigger and much more complex compared to the C. Elegans. Fluorescence microscopy helped to reconstruct the connection map of about 10% of all the neurons of the fly. The study of the projection map using graph theory has highlighted that its neuronal projection distribution is not asymptotic, but it has instead a heavy-tail, that it has a hierarchical modular organization within its sensory units, and that it has a small world characteristics associated within motor and auditory processes (Takemura, Nern et al. 2017).

In the mammalian brain micro-connectivity study has not been showed entirely yet, due to its larger connection range and its density. Many studies have tried to reconstruct a detailed map of wiring and targets. Since with the current tools it is a nearly impossible task, the common trend it to try to identify just specific connections (e.g. using retrograde axonal tracing with fluorescent markers (Schofield 2008)), or trying to label just specific cell type and see how

they are interconnected (McDonald 1992), or by trying to build a computational model to infer the wiring mechanism from the partial data that we have (Doya, Ishii et al. 2007).

Finally, nanoconnectomics studies the nano connections between pre- and post-synapses. This level of detail is achievable only using resolutions of nanometer scale. To try to identify the details of the synapses the most common used tool is the electron microscope. This technique uses a beam of accelerated electrons to excite a magnetic sample that in response scatters and emits electrons that are detected by a sensor. The detected electrons represent the shape and composition of the sample.

1.2.2 Neuron connectivity in vitro and functional studies

Another way to study the connections and communications that neurons establish is to observe them in vitro. Cell culturing is a very simple and reproducible way to observe the cells proliferate and interact without having problems related to optical access, density, etc. It consist of growing the specific cell line of interest in a controlled environment and at determined conditions, such as seeding material, media components, temperature, CO2 and oxygen concentration, etc.

The cells can be seeded on different materials and architecture. The most common way is to grow a layer of cells on a petri dish. The cells can either adhere on the bottom of the flat surface or they can need a coating to bind to. Using live fluorescent labels or fixing the cells and immunostain them at the desired time allows to see how a single neuron branches out to connect with other cells and create a network (Poli, Pastore et al. 2015). Using calcium imaging and patch clamping it is also possible to inspect how the pulse propagates among the connected cells and identify clusters. Nevertheless flat cultures of selected cell types are very different from neurons in vitro, witch are influences by surrounding non-neuron cells, vessels, Extra-Cellular Matrix (ECM) and other factors.

Other approaches are to try to culture cells in 3D, so in architectures that resembles more the in vivo organization (Frega, Tedesco et al. 2014). One is to use scaffolds and special materials to control the cell organization. This method is mainly used in a field called tissue engineering that is discussed in the next paragraph.

Other approaches are to cluster cells into aggregates that stick together to be in the less energetic state, or to grow 3D spheroids using stem cells, that organize themselves similarly to embryonic development.

Examples of the first approach are the neurospheres (NSPs) (Campos 2004). NSPs are aggregates of different types of cells that form a sphere shape when they can’t adhere on the provided surface. They can form from cell lines that don’t adhere unless the substrate is coated with an ECM protein, or if they are seeded on a hydrophobic material that prevents strong adhesion. They can be floating (Ladiwala, Basu et al. 2012), immobilized in

wells(Birenboim, Markus et al. 2013), or be semi-adherent on a surface and interconnect between different NSPs (Rabadan, Migliori et al., *In preparation*).

Usually they are formed by taking out specific parts of an embryonic brain, dissecting the pieces into cells, and seed them on the substrate. The cells then rapidly (overnight) organize into a more energetically efficient shape: a sphere.

If they are seeded on a hydrophobic surface with enough space, multiple NSPs can form. This model gives the possibility to observe both the long and the short-range connections among neurons both at structural and functional level. Infecting the cells to make them express GCaMP can show how the networks connect and interact and how a stimulus propagates from one cell to the whole NSP and then to the next.

On the other hand, brain organoids (BO) are spheroids that are grown from few stem cells and organize themselves into defined layers, partially mimicking the normal brain development (Lancaster, Renner et al. 2013, Di Lullo and Kriegstein 2017). The pluripotent stem cells are provided with growth factors that direct their fate towards a neuronal lineage and start to differentiate into all kind of brain cells (neurons and glial cells). Compared to the NSPs, BOs grows in size sensitively from the seeding day, and the growing rate depends on the species from where the cells come from. When the BOs reach a critical size, the media and its nutrients cannot reach anymore the core of the spheroid that, in absence of vasculature) starts to necrotize. Many researchers are now trying to provide some sort of artificial vessels or to find a way to deliver nutrients to the core, but still this is the main issue in the field. BOs are considered a great tool to be used in developmental biology and drug testing, given the possibility to use induced Pluripotent Stem Cells (iPSCs) for culturing. These cells are somatic cells that are turned back to a pluripotent stage thanks to genetic modifications (Takahashi and Yamanaka 2006). The great advantage of this discovery is that iPSCs can be retrieved in a non-invasive way, can be cultured, expanded and stored, and most importantly, can be taken from humans.

1.3 Tissue engineering and bioartificial systems

Tissue engineering is a field that aims to find a way to recreate as accurately as possible a specific tissue in order to replace a defective or missing one and restore biological functions (**Fig. 8**). A key factor to achieve this goal is to study how the tissue is organized and formed from the biological, chemical and material point of view. The new functional tissues are made of a combination of cells, engineered synthetic materials and appropriate bio-chemical stimuli.

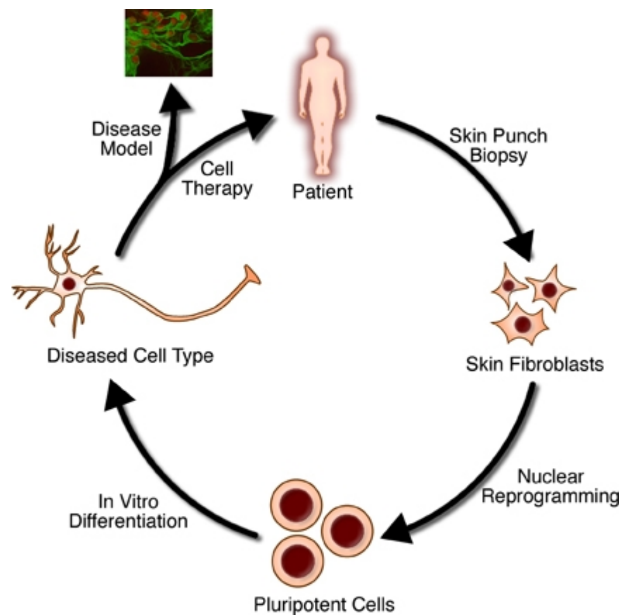

Figure 8 Schematics of the tissue engineering approach mechanism. (Rodolfa, K.T., Inducing pluripotency, StemBook 2008)

One essential component of most of artificial tissues is the support (scaffold) for the cells. These scaffolds have to provide both mechanical stability and an environment that mimics the in vivo ECM. For these reasons scientists chose the scaffolding materials taking into account their biocompatibility, stiffness, electrical properties, hydrophobicity, adsorption rate, degradation, etc. The materials more largely used are both natural and synthetic polymers. The natural polymers are often proteins taken directly from the ECM (gelatin, collagen, alginate, fibrin, self-assembling peptides, hyaluronic acid), so they show biocompatibility, weak inflammatory response in vivo and promotion of cell adhesion and proliferation. The disadvantages are that they show poor mechanical properties, rapid degradation kinetics and a poor reproducibility. The synthetic polymers (polyglycolic acid (PGA), poly (lactic-co-glycolic) acid (PLGA), polyurethanes (PU), polyglycerol sebacate (PGS), poly(ε-caprolactoneco-L-lactic) (PCLA), poly(N-isopropylacrylamide) (PIPAAm), polyvinyl alcohol (PVA), polyethylene glycol (PEG), poly (ε-caprolactone) (PCL)) show good workability, biocompatibility, degradability, tunable mechanical properties, but they are less biocompatible and not part of the ECM. Thermoplastic polyesters (PLA, PGA, PCL and their copolymers) are not suitable scaffold-forming materials for cardiac tissue engineering due to their high stiffness and poor strength to cyclic deformation. Elastomeric polymers (PU and PGS) show an elastic mechanical behavior that allows them to withstand significant deformation and recover its original dimensions when the stress ceases.

The combination of tissue engineering techniques with material science and electronics is also inspiring a new field that aims to mimic nature's structures and function to create biohybrid systems with specific functionalities. For instance, engineered living constructs able to control and coordinate tissue morphologies and functionalities are of high interest for designing state-of-the-art biorobots and biosensors useful in a wide range of applications (Kailashiya, Singh et al. 2015, Holley, Nagarajan et al. 2016, Park, Gazzola et al. 2016). Microfabricated cell-based microengineered actuators can greatly enhance the performance of biorobots, potentially resulting in cheaper, faster and easier-to-use analytical tools that are more portable and scalable for point-of-care sample analysis and real-time diagnosis (Lai, Sun et al. 2014, Hu, Cheng et al. 2015).

In addition, the combination of microengineered materials and biomimicry leads the technological effort of understanding and using the most successful results of natural

evolution to develop innovative methods, which can solve medical, environmental and engineering challenges (Chen, Ross et al. 2015, Green, Lee et al. 2015, Marino, Filippeschi et al. 2015, Zhang, Gu et al. 2015). In particular, natural mechanisms are generally sustainable, high performing and energy-saving, features that are key factors to create biological-based machines able to dynamically deform their shape and sense under specific environmental cues. For example, an entire research field, called biomechatronics, is focused on the study of biomimetic microactuators, where biological components, typically muscle cells, are integrated with a specifically designed artificial substrate (Ricotti and Menciassi 2012, Carlsen and Sitti 2014). The advantages related to the use of muscle cells have inspired the development of biomimetic constructs including muscular thin film based bio-hybrid actuators (Feinberg, Feigel et al. 2007), walking "biological bimorph" cantilevers (Chan, Jeong et al. 2012, Chan, Park et al. 2012), and self-propelled swimming robots (Williams, Anand et al. 2014). Usually, the natural element provides functional capabilities while the artificial substrate is used to efficiently exploit the properties of the biological components while ensuring good biocompatibility and improving their unique function (Herr and Dennis 2004, Kim, Park et al. 2007, Nawroth, Lee et al. 2012, Shin, Jung et al. 2013, Cvetkovic, Raman et al. 2014).

To create biomimetic hybrid actuator, microfabrication technologies are extremely powerful tools to achieve micrometric structures with high accuracy and repeatability. Soft photolithography is a set of microfabrication techniques that uses elastomeric stamps, fabricated from patterned silicon wafers, to print or mold materials at resolutions as low as several tens of nanometers (Xia and Whitesides 1998, Kane, Takayama et al. 1999, Ostuni, Yan et al. 1999, Ostuni, Chen et al. 2001, Khademhosseini, Jon et al. 2003, Suh, Khademhosseini et al. 2004). In addition, photolithography can be used to engineer the desired architectures (Whitesides, Ostuni et al. 2001, Walker, Zeringue et al. 2004), such as the body of micro-robots (Kim, Park et al. 2007), as well as micro-vasculatures within tissue engineered scaffolds (Kaihara, Borenstein et al. 2000, Borenstein, Terai et al. 2002) in a convenient and rapid manner.

Furthermore, the incorporation of nanomaterials into biomimetic hydrogels and the encapsulation of stretchable electronic components are demonstrated to be a suitable way to obtain the desired mechanical and electrical properties (Blum, Soto et al. 2011, Dvir, Timko et al. 2011, Shin, Bae et al. 2012, Rajzer, Rom et al. 2014, Shin, Shin et al. 2015, Baei, Jalili-Firoozinezhad et al. 2016). Even though the combination of biological materials with microelectronic components offers countless advantages, it is still very challenging to incorporate in effective and repeatable way the two parts together. As a matter of fact, electronic components and biological ones have completely different mechanical features, that make it very hard to cope with brittleness and fragility of the first with the high elasticity and low elastic modulus of the second. Additionally, since the structures are extremely small, it is very hard to create an electrical component at a micrometric scale that is also flexible and stretchable enough to be transferred on a different substrate and follow the bending of the "new host" without cracking or scratching.

2 AIMS OF THE THESIS

The overall aim of the thesis is to provide tools for studying connectivity of the cells from a structural and functional point of view.

Paper I: To propose a new microscopy tool for volumetric imaging of large tissues (Light Sheet Theta Microscopy, LSTM) to acquire large intact volumes of clarified tissues without compromising the image quality and the speed compared to existing light sheet microscopes.

Paper II: To describe a microscopy tool to investigate fluorescently labeled live cultures for prolonged periods of time, focused on limiting the light exposure on the sample and data handling in terms of online data extraction and data compression.

Paper III: To build a biohybrid actuator using the contraction of cardiomyocytes made of different layers of different materials that combined together provide mechanical stability, electrical pulses and induce cell growth directionality.

3 RESULTS AND DISCUSSION

3.1 Paper I

The recent development of tissue clearing methods is providing optical access of entire volumes of tissues. Nowadays the advances of these techniques combined with novel labeling methods are able to clarify and fluorescently tag increasingly larger samples. Furthermore, after the discovery of tissue expansion techniques is giving rise to the possibility of virtually increase the resolution of the objects at the cost of sample and data size. These discoveries can help to highlight and investigate the brain architecture at very high level of details.

However, to be able to image such large samples in a reasonable time frame and at micrometer resolution there is the need for suitable microscopy systems.

Light Sheet Microscopy (LSM) approaches have been proven to be effective for samples as large as a whole mouse brain. LSM's basic mechanism is to illuminate an entire plane of the sample using a thin sheet of light, and acquire it orthogonally using a CMOS camera. However, scaling up the sample size has been partly hindered by the use of orthogonal light-sheets. In fact the physical presence of the objective limits the size to a quarter of the objective's working distance. Furthermore, since the light needs to travel through the sides of the sample, each plane would suffer from light scattering in the middle portion of the slice.

We have developed Light Sheet Theta Microscopy (LSTM) to address some of these limitations. LSTM achieves planar imaging by rotating the illumination arms towards the camera side to free the lateral space of the sample. This configuration removes the limitations on the lateral dimensions of the sample, while providing similar imaging depth, uniform high-resolution throughout each plane and low photo-bleaching (higher than LSM for smaller samples, but similar to LSM for larger samples).

In LSTM a single frame is acquired by rapidly scanning a static light sheet over the plane (using a galvo scanner) and synchronously acquiring it using a CMOS camera in rolling shutter mode. Since the sheets are coming at an angle, to achieve uniform planar illumination it is necessary to translate the sheet in the axial dimension, to compensate for the focal plane shift as the sheet is scanning from one side to the other. This shift is achieved using an ETL (Electrically Tunable Lens), which can slide the focus accordingly to a different applied voltage. The whole volume is acquired by moving the sample using a three dimensional stage. The angle at which the two illumination arms are positioned is 60°, and it was decided by choosing a tradeoff between light sheet thickness and illumination path length, within the physically allowable range. The light sheet thickness influences the axial resolution of the system, while longer path lengths lead to more scattering and photobleaching.

In the paper there is an extensive characterization of the system and a comparison with a previous implementation of the lab: COLM (Clarity Optimized Light-sheet Microscope), which was assembled using the exact same optical components. LSTM keeps the same speed

of COLM since both the ETLs and the Galvo scanners are much faster then the camera speed. Furthermore the component that is slowing down the whole acquisition is the stage. In terms of image quality, both systems provide nearly the same resolution in the 3 axes. The main quality difference is related to the illumination path. If the sample is large and flat, the light would have to travel through the entire lateral sides to reach the center in the COLM case, which leads to heavy scattering and photobleaching. Nevertheless if the sample is small and bulky, LSTM would have a longer travel path for most of the volume, especially in the deeper layers. Furthermore redundancy would be much higher compared to the COLM system, leading to higher photobleaching.

In the paper we present multiple examples of volumetric acquisitions of mouse, rat, human and Hydra nervous systems. We used Thy1 genetically modified mouse brains, Central nervous systems and expanded slices to show the architecture of stem cells and mature neurons in the brain. This label is perfect to show the microscope capability, since it is sparsely present in the brain and is largely diffused. The brain samples were clarified using the CLARITY technique, and imaged using 10X and 25X objectives, corresponding to respectively 11,11 and 27.78 effective magnification (we used tube lenses from Thorlabs with 180 mm of working distance and objectives from Olympus, that are supposed to be paired with tube lenses of 200 mm of working distance), and a resolution of 0.585 and 0.234 μm per pixel respectively. The expanded sample was 4 times bigger than the original slab of tissue, meaning that every labeled component had a virtual resolution 4 times higher. This specific sample would have been impossible to image using the COLM, and would have taken several days of continuous imaging using a point scanning microscope (e.g. Confocal). Using the LSTM system it only took 22 hours.

In addition, we imaged a large slab of CLARITY clarified rat brain labeled for vasculature (Lectin), and a chunk of human brain labeled for nuclei (DAPI). Both these samples are characterized by broad labeling and high tissue density (especially for the human sample). These characteristics make the scattering issue more evident, since the light have more difficulty to travel through the sample. Indeed, we used the rat slice to make a penetration comparison between the COLM and LSTM.

Finally, we performed live volumetric imaging on a genetically modified GCAMP hydra in free motion. This little transparent animal undergoes drastic changes in its body and cellular density. These radical variations would need an adaptive mechanism to change the offset of the laser accordingly to each movement, if illuminated orthogonally. Using LSTM though the laser path is much shorter and does not need that compensation, as it is shown from the traces generated from the calcium signaling of tracked cells.

3.2 Paper II

The advancement of microscopy technologies and labeling techniques is leading to higher definition of the images and higher speed of acquisition. These amazing new results though usually come with an increase of the datasets size and related difficulties in data handling.

With the advent of dynamic tags that can show live events in living tissues (eg. calcium indicators), microscopy systems need to acquire bursts of images at very high speed and for long periods of time. This leads to the problem of storing the images during the experiment time and bulk processing of large datasets once the experiment is over.

In this paper we propose a microscopy system (called XDscope) designed to perform long term monitoring of living cultures using fluorescent dynamic dyes, with a particular focus on data handling and analysis. From the hardware point of view, the system is designed to be small enough to fit multiple of them in an incubator, to be cheap in order to be able to perform multiple experiments at the same time, to have a large cheap to image large portions of the sample without needing a stage and to minimize as much as possible the laser power. From the software perspective, the control and the GUI (Graphical User Interface) were made to be simple and easy to use, but still to leave as much freedom as possible to the user in terms of data resolution, compression and ROI (Region Of Interest) selection. The main features of the XDscope are that it can perform a pre-acquisition at the highest possible resolution and low speed to capture all the spatial features, acquire long and fast bursts coupling multiple cells to create one pixel (this both reduces the data size and enhances the sensitivity of the camera), select automatically ROIs from the high resolution images (with manual feedback), and use the ROIs map to plot (and the relative file) of the signal overtime for each timepoint and to sparse compress the data for storage (up to 200 folds smaller than the original image).

This system is particularly suitable for the long term monitoring of GCaMP-expressing neurospheres (NSP) or organoids, since the majority of the cells are clustered together leaving large portions of background. To assess the validity of the system, we imaged for 2 weeks an NSP network sample infected with GCaMP6. This particular preparation that we used is made of cells isolated from the hippocampus and seeded on a hydrophobic surface. In these conditions the cells arrange into spheres that are then interconnected among each other. Infecting them using GCaMP gives the possibility to observe how the cells send and process the signals both at neuron and neurosphere level. In paper 2 we show an example of an output from the first timepoint of an experiment performed on a neurosphere network derived from wildtype mouse, and the relative detected spikes over 10 hours of recording.

In addition, we used the XDscope to assess the uptake mechanism of a specific LCO called p-HTMI designed to target immature neurons and stem cells. We performed 30 minutes of continuous recording of the NSP network with applied p-HTMI. We then observed that after around 10 minutes from the application p-HTMI starts to label progenitor cells (which are normally located in the outer part of the sphere), and after 25 minutes it seems like most of the molecule from the media goes inside the targeted cells.

To further check what the target of p-HTMI is within the cells, we performed double labeling of the molecule with putative target starting from ER markers and Golgi markers. The

resulting high magnified images and stacks suggest that p-HTMI is most probably targeting the molecule Golga2, which is responsible for facilitating the transport between the ER and the Golgi.

3.3 Paper III

Engineering hybrid living-synthetic biorobots with the capability to actuate in a biological environment is of interest for biomedical applications such as building biorobotics and biosensors. It is also a way to study and try to replicate the mechanisms underlying the fisyological functioning of living organisms. Specifically, the way the cells arrange themselves in a network is very important for the functionality of the tissue, together with the mechanical, electrical and chemical properties of the substrate.

Here, we report the development of a bio-inspired hybrid living construct with integrated self-actuating cardiac myocytes. The cells were seeded on a micro-fabricated hydrogel scaffold embedding a stretchable gold (Au) microelectrode. The shape and layer construction was inspired by the Manta ray. Their relatively simple shape and their way of swimming by flapping their wings motivated the three layers and the induced arrangement of the cells. The first layer was designed to mimick the backbone of the Manta. The architeture and the stiffness of the chosen material (PEG (PolyEthylene Glycol)) were designed to release the tension after each contraction, and at the same time to allow the bending of the wings.

The layer in contact with the cells was designed to be biocompatible and to promote the cell growth in a preferred directions. Furthermore it had to be electrically conductive and mechanically resistant to sustain the beating cycles. For these reason we designed a CNT-GelMA (Carbon Nano Tubes Gelatin methacryloyl) substrate layer with patterned grooves to align the cells in the preferred contraction direction. The grooves are spaced enough to align the cells but also allow the cardiomyocytes to connect between each other in the upper layer, so that the contraction is strong and propagats from the center of the structure toward the tip of the wing. To further control the propagation and strength of the pulse, we integreted a gold electrode between the layers.

The resulting bio-inspired robot showed excellent myofiber organization and unique self-actuating movement, which was directly related to the direction of contractile force of muscle tissue. Stretchable Au microelectrode allows local electrical stimulation and tailored control on the beating behavior of the bio-inspired robot. Therefore, the proposed cardiomyocytes-based living construct can strongly contribute to enhance the current understanding of how cell-based microengineered actuators can be efficiently controlled and tuned through customized local electrical stimulation. This study can contribute to the construction of customised cardyac patches for a more physiological integration of engineered tissue costructs.

4 CONCLUSIONS

The work described in this thesis aims to provide different tools for the study of cells connections in terms of architecture and function.

The first described tool is a microscope (called LSTM, Light Sheet Theta Microscopy) that allows the volumetric imaging and mapping of whole optically cleared organs. This method can show in a single acquisition how the cells interconnect and arrange in a tissue. Since the brain is a very complex organ with a lot of different functionalities that are not yet fully understood, we tested the microscope mainly on the central nervous system labeling neuronal subpopulations. The examples that we illustrate in this paper show that the microscope has the capability to image large samples with homogeneous quality throughout each plane, in contrast to conventional light sheet microscopy (LSM), which experience an increasing scattering in the central portion of the sample when the lateral dimension is large. Other systems with illumination coming from the same size of the camera (e.g., confocal, 2 photon, etc) impart a high light exposure on the sample leading to photobleaching and is very slow compared to LSM systems. LSTM on the other hand is as fast as LSM and with the same image quality, but it is overcoming the LSM geometrical limitations on the lateral size of the sample and is avoiding the scattering issue in the x-y plane, leading to uniform resolution and intensity throughout each plane. This method can be used to trace long-range connections and study how different areas are linked. The long-term goal is to use LSTM to acquire and map the whole human brain (slab by slab) in a reasonable amount of time.

Together with the physical connections, studying how cells communicate within a network is crucial for the understanding of functional mechanisms. An effective way to study how the depolarization propagates is to observe calcium signaling in living tissues. Long recordings of live cells expressing fluorescent photons upon the release of calcium signaling is a very spread way to inspect how the cell communication evolves overtime. This is though not trivial both from the healthiness of cells perspective, and from the big amount of data to store and analyze, posing restrictions on the acquisition duration.

Hence, the second tool we developed is an integration of a microscopy system with a processing semi-online algorithm for recording and data handling of long term live imaging acquisitions. The main features are the small size, the low cost, the customizability of resolution, the calculation and plotting of the intensity of the segmented objects, and the following sparse compression. The whole system was designed to limit the user manual interaction with the culture (limiting the times the incubator is open too), and to provide rapid assessment of the result of the experiment and limit the size of the data collected without compromising the quality of the information. The level of compression and automation can be set by the user, resulting in an easy way of controlling the experiment settings. This system was assessed by performing weeks long recording of an NSP network. Furthermore we inspected the uptake mechanism of an LCO library (called p-HTMI) performing live imaging of a NSP network continuously for 30’. At last, we complemented the study of p-

HTMI using a confocal microscope to understand the specific target of this molecule within the cell. The results suggested that the molecule is targeting Golga2, which is a protein that aids the transportation between the ER and the Golgi apparatus.

Another way to understand what are the mechanisms behind the functionality of a tissue is to try to reproduce its behavior by mimicking all its components to create a construct with similar features. In particular, controlling the way the cells arrange themselves, interconnect in the different layers, and propagate their pulse within the network is crucial for the functioning of both brain tissue and cardiac tissue.

To try to recreate features of the cardiac pump contracting, we fabricated a micro-engineered hydrogel-based living construct based on neonatal cardiomyocytes cell. The cells are seeded on a CNT-GelMA pattern overlying a PEG patterned layer with a gold microelectrode incorporated between the two materials. Mechanical and optical characterizations of the structure showed stability and ideal conditions for cells organization and maturation. High viability during the seeding (day 0 of culture) was followed by spontaneous beating synchronization along CNT-GelMA pattern (from day 3 of culture until day 7 of culture). Staining after day 5 revealed homogenous F-Actin distribution and partial uniaxial alignment of cells along the CNT-GelMA pattern. The electrical characterization of the biohybrid actuator was performed both using external carbon rod electrodes as well as embedded micrometer-size gold electrode. Although *in loco* stimulation featured higher excitation voltage thresholds with respect to the external one, it was possible in both cases to control the beating up to a frequency of 2 Hz regardless of the natural beating frequency, by applying voltages lower than 3 V. The valuable results obtained in the present work not only encourage further developments in the field on biohybrid actuators but also underpin new cutting-edge studies about the *in loco* control of the electrical stimulation of such scaffolds embedding customizable microelectrodes that can also potentially lead to establish a wireless control of the whole system. Furthermore this approach can be further explored to build customized cardiac patches or scaffolds for heart tissue regeneration.

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