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Development and Activity of *In Vitro* Neuronal Networks

Learning Organic Chemistry through Games

Par Maéva Vignes

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Dirigée par Jean-Louis Viovy

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Devant un jury composé de :

Catherine Villard, DR	Rapporteur
Elisha Moses, Prof.	Rapporteur
Samuel Bottani, MCU	Examineur
Martin Oheim, DR	Examineur
Jean-François Le Maréchal, MCU	Examineur
Jean-Michel Peyrin, CR	Examineur
Jean-Louis Viovy, DR	Directeur de Thèse
Bernard Brugg, CR	Co-directeur de Thèse



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*“Impose ta chance, serre ton bonheur, et va vers ton risque,
à te regarder, ils s’habitueront.”*
René Char

Introduction: short story of my PhD

The roots of the initial project...

Few years before I started my PhD a collaboration was initiated between the team of Bernard Brugg and Jean-Michel Peyrin at Univeristy Pierre et Marie Curie (UPMC), studying neuro-degenerative processes, and the team of Jean-Louis Viovy at the Institute Curie, developing biomedical applications of microfluidics systems. Microfluidic devices that can compartmentalize the cell bodies and the axons of neurons were developed. Using special funnel-like channels, the first two-compartmented (2C) oriented network with cortex neurons (Cx) connecting striatum neurons (St) was built.

...The trunk of the project...

At the beginning of my PhD, I sat up hippocampal cultures in the laboratory of Bernard Brugg and built the second 2C oriented neuronal network with Cx connecting hippocampus neurons (Hi). The reconstruction of this novel network had the double interest of generalizing the techniques and offering a new platform for neuro-degenerative studies.

I also begun to explore diverse geometries of micro-channels to orient axonal outgrowth and control the orientation of synaptic connections in microfluidic chips.

...Meandering in the dendritic tree...

In the lab I explored a lot of topics that will not be all presented in this manuscript. I tried to embed collagen gels or matrigel in our chips, begun studies about peptides involved in Alzheimer's disease, did some preliminary studies to control flows in the microfluidic devices with a pressure controller. I supervised Benjamin Lassus during his master internship to develop micro-patterning techniques.

In my doctoral school I got involved in many activities. I co-organized the second Paris interdisciplinary PhD symposium (PIPS 2011) on “Shapes in Living Systems” (<http://symposium.fdv-paris.org/2011>). I actively participated and co-organized scientific clubs, alternating journal clubs, seminars and brainstorming sessions, on Neuro and Optics and “In Vitro Artificial Intelligence” (IVAI). I participated in an Researcher-Journalist exchange organized by the french Ministry for Higher Education and Research. At this occasion I welcomed the journalist Sophy Caulier for one week in the lab, and later worked for one week in the editorial team of “Pour la Science” (french equivalent of “Scientific American”). I volunteered in the Paris Montagne association, welcomed highschool students in my lab for one week internships, and yearly helped to organize the science festival of this association. Finally I taught in the chemistry team of the “Frontiers in Life Sciences” bachelor program.

I must admit that all these parallel activities might have been overwhelming sometimes. However, through all of them, I managed to enrich my scientific experiences and ideas, and tried to share my curiosity for sciences, and my pleasure to do research.

...Integrating inputs to build new topics...

All these diverse interactions and explorations lead me to deviate a little from the first objectives and projects of my PhD. Thanks from the freedom that my supervisors gave me, and through many discussions with my colleagues inside and outside of my labs, I managed to develop projects that were more reflecting my own interests. Three topics emerged that will be presented in this manuscript: (1) development of a semi-quantitative method to compare different micro-patterning techniques used to build neuronal networks *in vitro*, (2) development of a fully optical method to stimulate and record neuronal cultures activity, and (3) a preliminary study of the pedagogical impact of games to learn basic concepts of organic chemistry.

Organization of this manuscript

My manuscript is organized in two parts and 5 chapters. The first and main part present my work on *in vitro* neuronal networks at the frontier between biophysics and microfluidics. The second part present a project in pedagogy involving the creation, use and preliminary evaluation of four games used to teach chemistry to undergraduate students.

The first part on *in vitro* neuronal networks is divided in four chapters:

- The first chapter comprise basic concepts that are useful for an interdisciplinary reader to understand the studies that follow. It also sketches a brief state-of-the-art of the emerging field of micro-neurobiology, at the crossroads of micro-technologies and neurobiology.
- In the second chapter, the general interests and challenges to build neuronal networks in microfluidic systems are highlighted. A first proof of concept of the possibility to build a neuronal network mimicking the physiological interactions between cortex and hippocampus is explained.
- The third chapter quantitatively compares three micro-patterning techniques in their ability to position cell bodies, orient neurites outgrowth and differentiate between axons and dendrites.
- Finally the fourth chapter describes a novel fully optical method to stimulate and records the activity of neuronal networks *in vitro*.

The second part is more condensed and describes the pedagogical aims of the games and their rules. A preliminary assessment of the pedagogical impact of these games is also presented.

Finally the last chapter sum up the main results and conclusions and opens on my future research projects.

Part I

Development and Activity of *In Vitro* Neuronal Networks

“Je ne travaille pas, je bricole...”
Papy Henry

Chapter 1

At the crossroads of Neuronal Networks and Microfluidics

This chapter contains the literature of an emerging field at the frontier of neuroscience and microfluidics. This state-of-the-art is non-exhaustive but will help to position my contribution to this field. This chapter will also provide some basic notions in neurobiology and microfluidics to help the reader to understand my PhD work.

1.1 Neuronal Networks and Microfluidics

What are neuronal networks?

Since Golgi and Ramón y Cajal work, we know that our brain comprises “morphological entities” called “nerve cells”, showing “dendritic or protoplasmic processes”, that “possess reciprocal relationships” with other nerve cells “*in contiguity* but not *in continuity*” [1]. Though, neuronal networks are assemblies of individualized nerve cells (or neurons) that communicate through tight gaps called synapses.

Neurons convey and process information in our brains. Neurons have a singular morphology linked to their function. A typical neuron shows (i) a round cell body, (ii) a dendritic tree (ramification of cell processes called dendrites) that collects informations from afferent neurons, and (iii) a large process (typically several millimeters) called the axon that convey information to the next neurons (fig. 1.1).

Different neuronal sub-populations exist that show slightly different morphologies and properties (fig. 1.2). When the axon of a neuron and the dendrite (or cell body) of another neuron enter in close contact, communication points or synapses are formed. A neuron can form approximately 10000 synapses with its neighbours. We can then imagine the puzzling complexity of the communication pathways built in our brains that comprise around 10 billion of neurons [2–4].

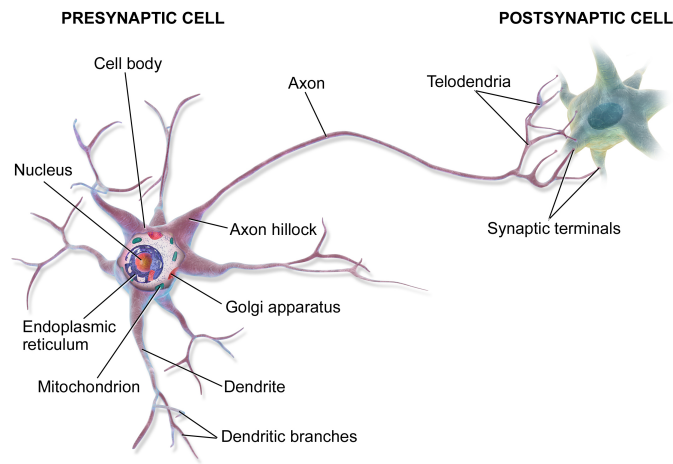
Fortunately, these communication pathways are far from random at large scales. The synaptic connections of our neuronal networks are the result of the controlled development of our brains. During this development, sub-populations of neurons are guided towards their physiological targets and false connections are eliminated [5]. At the end of this development, the mapping of the main communication pathways of our brains is set and will be more or less conserved (fig. 1.3). Nevertheless, at smaller scales, the architecture of small neuronal networks and their synapses will evolve continuously, allowing us to think, learn and memorize. This process is known as plasticity and occurs at the level of neuronal populations, individual neurons and synapses [6].

Motivations and challenges to study neuronal networks *in vitro*

Neuronal networks support our cognitive functions [2]. They have been extensively studied *in vivo* and mimicked *in silico* by artificial neural networks [6–11].

Classic experimental models used to study neuronal networks range from whole animal models, which preserve the anatomical structures but greatly limit the experimentation at the cellular level, to dissociated cell cultures, that allow detailed manipulation of cell phenotype but lack the highly ordered and instructive brain environment. Thus new experimental models are needed to facilitate both individual cell manipulation and brain connectivity reconstruction *in vitro* (fig. 1.4).

Controlling the topology of neuronal networks would open new routes to test hypothesis that cannot be tested *in vivo* or *in silico*. To the computer scientists, it would give the opportunity to investigate the behaviour of living neuronal networks with artificial and controlled topographies. To the neurobiologists, it would give a unique tool to mimic the development, activity or degeneration of physiological networks. I will discuss in the chapter 2 how *in vitro* neuronal networks can be used to study Alzheimer’s disease.



The Anatomy of a Multipolar Neuron

Figure 1.1: Illustration of the typical morphology of a multipolar neuron. *Credit: Bruce Blaus, Wikimedia Commons, <http://en.wikipedia.org/wiki/Neuron>.*

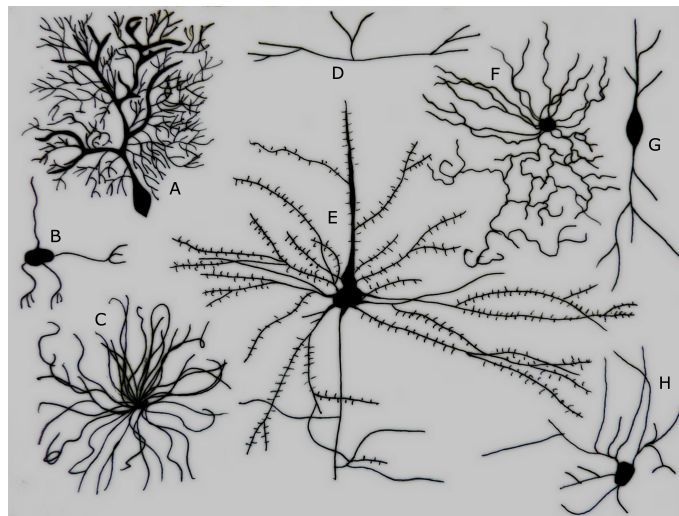


Figure 1.2: Illustration of different morphologies of neurons, adapted from the work of Santiago Ramón y Cajal. A. Purkinje cell B. Granule cell C. Motor neuron D. Tripolar neuron E. Pyramidal Cell F. Chandelier cell G. Spindle neuron H. Stellate cell. *Credit: Ferris Jabr, based on reconstructions and drawings by Cajal, Scientific American <http://blogs.scientificamerican.com/brainwaves/2012/05/14/know-your-neurons-the-discovery-and-naming-of-the-neuron/>.*

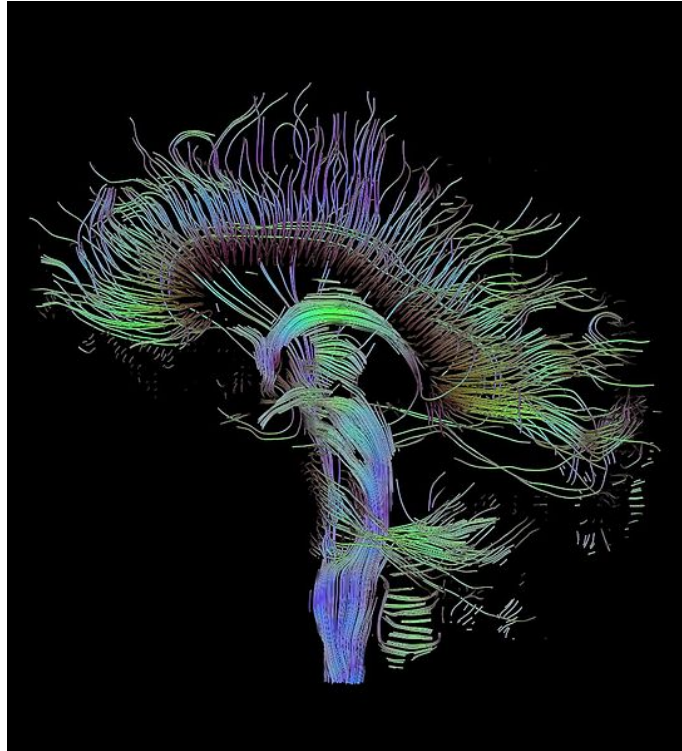


Figure 1.3: Visualization of a DTI (diffusion-tensor-imaging) measurement of a human brain. Depicted are reconstructed fiber tracts that run through the mid-sagittal plane. Especially prominent are the U-shaped fibers that connect the two hemispheres through the corpus callosum (the fibers come out of the image plane and consequently bend towards the top) and the fiber tracts that descend toward the spine (blue, within the image plane). *Credit: Thomas Schultz, University of Utah, Wikimedia Commons, <http://en.wikipedia.org/wiki/Connectome>.*



Figure 1.4: Main advantages and limitations of an *in vitro* approach as compared to an *in vivo* approach.

Controlling the topology of neuronal networks implies controlling communication pathways or synaptic connections between different populations of neurons. The main challenges are then:

- to organize the different populations of neurons,
- to guide the growth of dendrites and axons,
- and to control where synaptic connections will occur.

The fast growing research field of microfluidics offers new tools to biologists to develop interesting cell culture systems allowing handling cells in micron size environments.

What is microfluidics?

At the end of the XXth century, technological advances in miniaturization have enabled the development of new techniques grouped under the name of microfluidic systems or lab-on-chips. Microfluidic systems allow automatic and reproducible handling of micro-volumes of fluids through a network of channels and chambers of micrometers size (fig. 1.5). By analogy to microelectronics, microfluidic systems can be compared with microprocessors, where electrons and circuit tracks are replaced respectively by molecules or cells, and micro-channels [12].

Microfluidic systems are interesting platforms for various bioanalytical and biological applications. Many biological and biochemical techniques, such as electrophoresis, DNA sequencing, cells counting or sorting, or cell culture, have been miniaturized using microfluidic systems [13]. Miniaturized systems offer many advantages over their macroscopic counterparts: reduced volumes, smaller biological

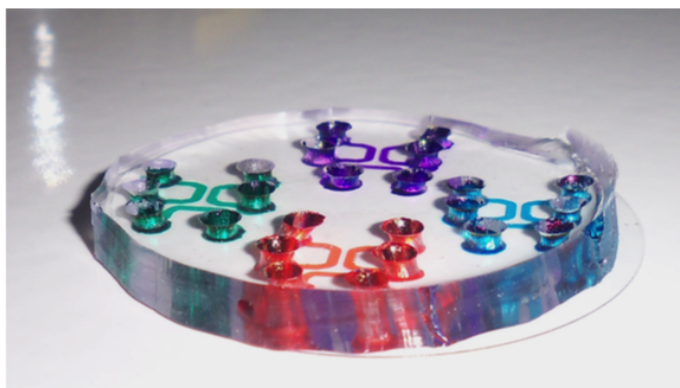


Figure 1.5: Example of a PDMS microfluidic chip showing four microfluidic circuits comprising each three main micro-channels colored by inks. *Credit: Deborah Dos Santos, Science Ac' welcomed for an internship in the lab..*

samples used, decreased reaction time, low cost, low power consumption, easier prototyping, high-throughput, better portability and increased reproducibility [14].

At the micrometer scale, the physics law of hydrodynamics are singular. The confinement of liquids in micro-channels implies low Reynolds numbers (meaning that the viscous forces are dominant compared to inertial forces). Flows are then laminar and different string of liquids can be flown in the same channel without mixing at short time scale (fig. 1.6). Molecular transport in between co-flows is induced by diffusion that can be used to create concentration gradients in micro-channels. In microfluidic systems, the ratio between surfaces and volumes is unbalanced and surfaces effect are dominant. This feature can be an advantage to detect rare molecules in biological samples. However, it can also be problematic in cell culture applications if molecules of interests (either injected in the system or secreted by the cells) are on the surfaces. An example of difficulties of immunostaining is shown in figure 1.14.

Polydimethylsiloxane or PDMS, polymer of chemical formula $-(\text{Si}(\text{CH}_3)_2\text{-O})_n-$, has emerged as the material of choice for biological applications of microfluidic systems (fig. 1.5). This polymer is biocompatible, transparent, very little permeable to water but permeable to gas, show low intrinsic fluorescence, and it can be easily shaped by soft-lithography techniques [16]. This material is advantageous for microfluidic applications as it provides low-cost, simple and rapid way to develop integrated devices. Other materials, such as thermoplastic materials (COC, PMMA, ...), are used for microfluidic applications. These materials allow higher throughput but are less convenient for prototyping, thus they are used when technologies are mature enough or for industrial applications.

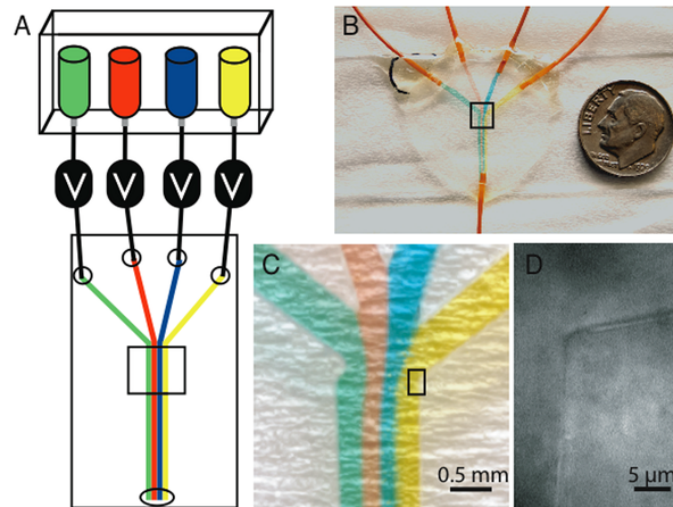


Figure 1.6: When multiple solutions are added into the same microchannel, they flow in separate flow lanes (no mixing) due to laminar flow characteristics. *Credit: Cousron and Rock, Wikimedia Commons, from Courson and Rock, Plos One, 2009 [15].*

Micro-patterning techniques for cell cultures

Microfluidic systems allow to control cellular environment. To control the position and organization of mammalian cells in culture, micro-patterning techniques, that were developed in parallel using lithography and soft-lithography techniques, are very useful.

Neurons are traditionally cultured on glass or polystyrene homogeneously coated with adhesive molecules, such as fibronectin, laminin or poly-lysine. Such coatings have been extensively optimized for each type of cells allowing good survival and growth of neurons *in vitro* [17, 18]. Such cultures, however, are mostly randomized in 2D. They are lacking the very accurate and specific spatial organization that characterizes multi-cellular organisms. Microfluidic and soft-lithography allow micro-patterning of environments in 2D or 3D to better recapitulate physiological organization.

I present here the most common techniques to pattern surfaces in 2D or 3D for cell cultures. For more exhaustive and detailed review, refer to Nakanishi et al. or Falconnet et al. for 2D techniques [19, 20], and Nikkhah et al. for 3D techniques [21], or Verhusel et al. for both [22]. The main techniques used in 2D and 3D patterning are similar though they are used differently to obtain either chemical or topographical patterns.

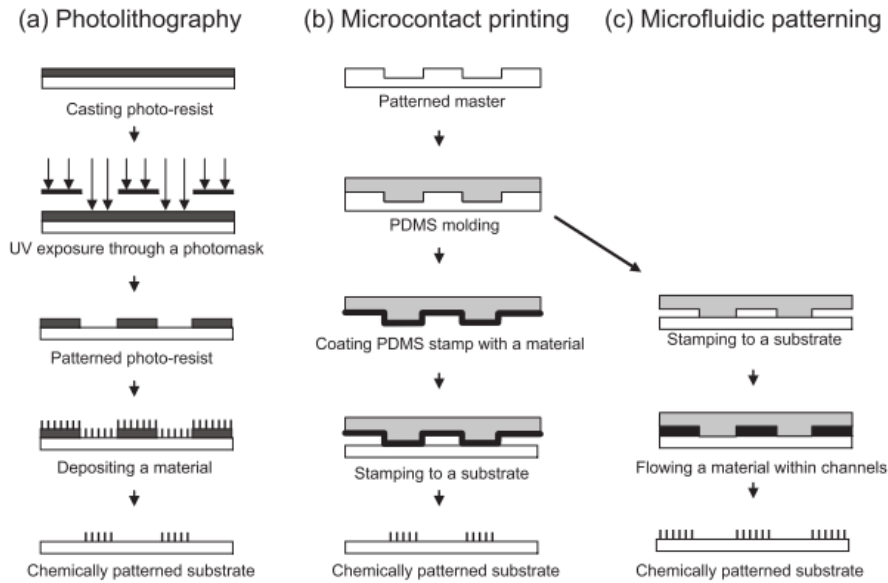


Figure 1.7: Illustration of three 2D micro-patterning techniques. *Credit: Nakanishi et al. [19].*

The goal of 2D micro-patterning techniques is to chemically pattern flat surfaces to trigger cell adhesion or repulsion in different areas (fig. 1.8). Depending on the size of the patterns, they can be used to shape the cells or to create a controlled pattern of one or several populations of cells.

- Photolithography was the first method employed. In this method a surface (inorganic material, mostly silicon, glass or metal) is uniformly coated with a resist that is sensitive to UV light. Under UV illumination through a photomask the resist is either destroyed or activated selectively in the transparent regions of the photomask. After illumination, a development step allows to remove selectively the destroyed or non-activated surfaces (fig. 1.7). Eventually, a second coating step is performed allowing a dual coating of surfaces. The limit of resolution of this method is triggered by diffraction and corresponds to the wavelength of the light used (typically few hundreds of nanometers). To go beyond this limit electromagnetic radiations with lower wavelength (X-ray, electron ...) can be used instead of the combination of UV lamp and mask.
- Photochemistry is also used to generate 2D patterns [23]. In this case UV light is used to trigger a chemical reaction of the substrate that creates the pattern. If a laser is used to scan the surface to coat the technique is then often called laser patterning.
- Soft-lithography techniques that are more flexible than photolithography, and allow rapid prototyping and high-throughput, are nowadays widely used in

biology, especially for cell culture applications [13]. Soft-lithography methods require the fabrication of a master by photolithography. This master is then used to mold or emboss an organic material. PDMS is the most used material for molding. COC is an example of thermo-plastic material used for embossing. In the broad range of soft-lithography 2D micro-patterning methods the two most used are micro-contact printing and microfluidic patterning.

- Micro-contact printing use a structured soft material as a stamp to deposit molecules on specific areas of a surface [24] (fig. 1.7). The stamp is usually obtained by PDMS casting. The transfer of molecules from the stamp to the surface is only possible if these molecules have a stronger affinity to the substrate than to the stamp. Spatial resolution and high reproducibility can be challenging with certain cue/substrate combinations. The principal characteristics and limitations of micro-contact printing are very well presented and detailed in the review from Perl et al. [25].
- Microfluidic patterning uses micro-channels to flow a coating agent only on some areas of a surface (fig. 1.7). Once the coating is performed, micro-channels can be disassembled [26].
- Micro-contact printing and microfluidic patterning can also be combined to achieve dual patterning of a surface [27]. PDMS removable stencils can also be used to coat a surface on controlled uncovered areas [28].

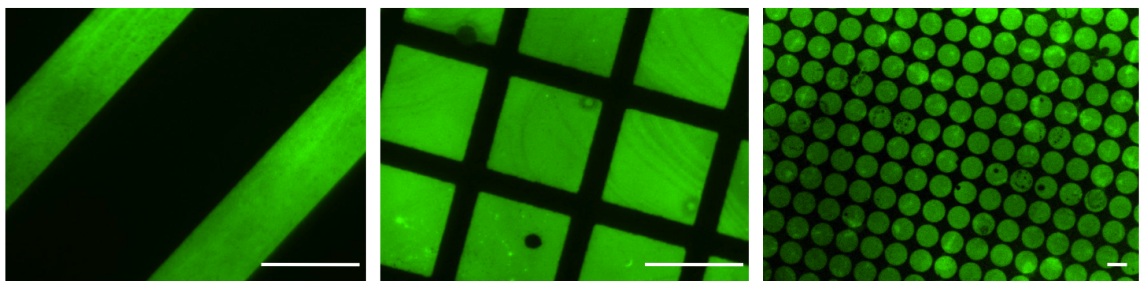


Figure 1.8: Examples of different geometries of micro-contact printing of poly-lysine labeled with FITC. *Scale bars* = 40 μm

The goal of 3D patterning techniques is to obtain structured surfaces with a controlled topography that can be chemically patterned. A wide range of techniques can be used to obtain randomly structured micro-topographies but we will focus here on techniques that create ordered micro-fabricated structures (fig. 1.9).

- Photolithography can be used again. To obtain microstructures, the surface of a wafer is coated by a photosensitive resist with a controlled height. The photoresist can either be laminated on the surface, if it is in the solid phase (as a dry film), or spin-coated if it is in the liquid phase. UV light illumination

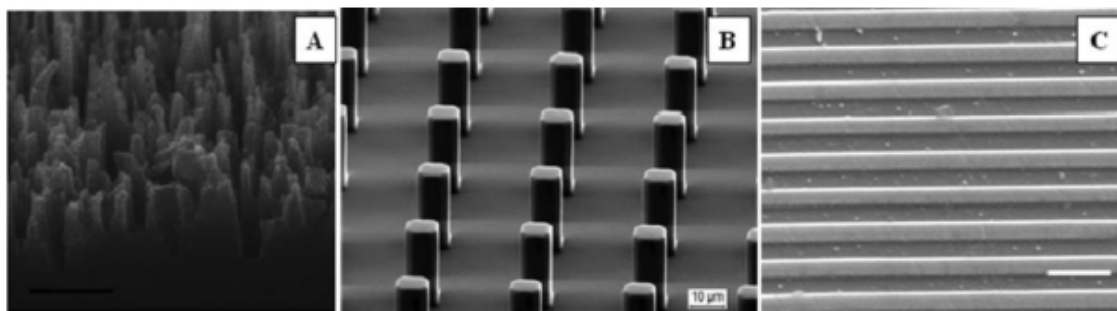


Figure 1.9: Examples of different kinds of topographical modifications. (A) Nanoscale roughness on silicon (scale bar 20 nm); (B) pillar structure on silicon (scale bar 1 μm); (C) parallel grooves on PDMS (scale bar 5 μm). Credit: (A) Turner S. et al., 1997; (B) Turner A.M. et al., 2000; (C) Gomez N. et al., 2007. Images from Khan and Newaz, 2010 [29].

through a mask is again used to reticulate or destroy the resist (respectively negative and positive resist) in specific areas. After development, well defined and rigid micro-structures are obtained.

- Stereolithography and two-photon absorption lithography are used when the resolution of photolithography is challenged. These techniques are mask-free and use a UV laser to cure the resist.
- Soft lithography is often used to create soft and/or curved topographies not accessible through photolithography [13]. The step of casting or embossing described above is used. In some specific cases micro-molded surfaces can also be chemically patterned to restrict the adhesion of cells. For example the in-mold patterning technique allow to mold a plastic while coating specifically the bottom surfaces of the structures with an adhesive molecule [30] (fig. 1.10).

In my work I used three micro-patterning techniques: micro-contact printing of an adhesive molecule called poly-lysine (2D technique), micro-grooves molding and in-mold patterning (two 3D techniques). In the chapter 3 I will compare these three techniques regarding their ability to build neuronal networks *in vitro*.

1.2 At the frontier of Microfluidics and Neuroscience

Microfluidics and microfabrication techniques has been recently applied to neuroscience, allowing new studies in neuronal development, diseases and pharmacology [31]. I will focus here on the development in microfluidics and microfabrication that has led to the reconstruction of oriented neuronal networks *in vitro*.

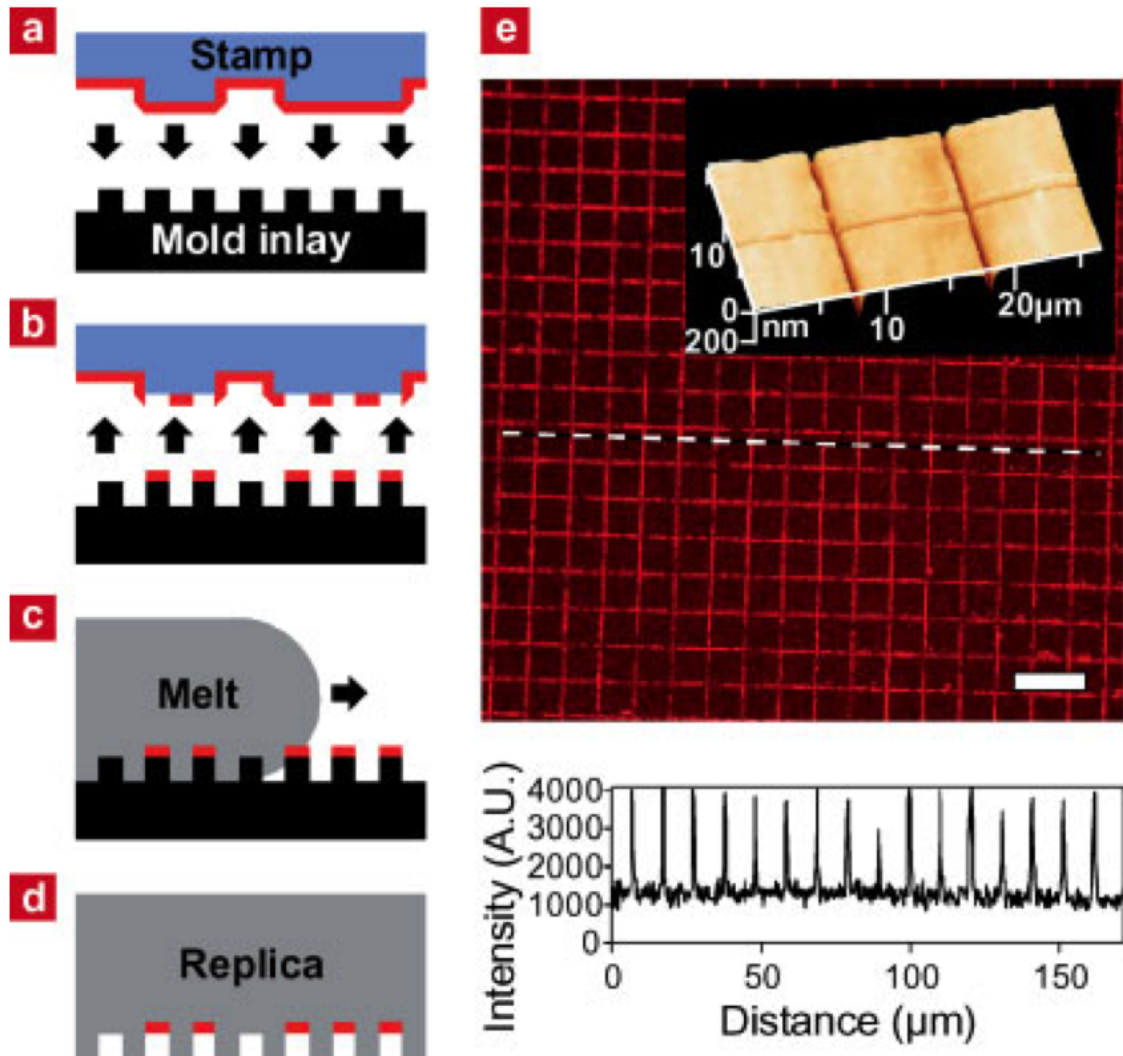


Figure 1.10: Principle and example of in-mold patterning. (a–d) Outline of the in-mold patterning process. (e) Fluorescence microscopy of fluorescently labeled antibodies transferred onto a PMMA replica by injection molding. The intensity profile reveals a grid line spacing equal to that of the mold inlay surface relief. Insert: atomic force microscopy of the replica surface shows an equivalent grid of trenches consistent with the transfer of antibodies to recessed surface areas. Scale bar 20 μm . Credit: Biancardo et al., 2008 [30].

Micro-patterning techniques guiding neuronal outgrowth

2D micro-patterning of surfaces has long been used to control the orientation of neurite outgrowth [31–33]. The study of Letourneau showed that neurites outgrowth could be restricted on palladium, collagen, or glial cells coated surfaces with increasing efficacy [32]. The study of Baier and Bonhoeffer [33] highlighted the fact that axons has a tendency to grow in the direction of increasing gradient of guidance molecules.

Significant progress has been made towards the precise control of dendrites vs axons elongation. The mechanisms at play in neurites outgrowth are better understood [34,35]. These mechanisms revealed the crucial role of the growth cone, scanning surfaces to find the best path for axonal outgrowth. When neurons are growing, neurites are not differentiated at first, the differentiation of one of the neurites into the axon is happening after 2 to 4 days *in vitro* [36,37]. The mechanisms of axonal polarization can be controlled *in vitro* by:

- Specific growth factors, in solution [38] or deposited on surfaces [39],
- Adhesive (or guidance) molecules gradients [40–42],
- Specific geometries of adhesive patterns [43,44].

The very recent and interesting work of Hart et al. [44], studying the behavior of neurons seeded on poly-lysine striped surfaces, with varying stripes width and gap length, suggested the importance of size patterns in accordance with the growth cone scanning area to better control neurite outgrowth direction.

Studies from Bonhoeffer team and Offenhäusser team highlighted the fact that, if axons grow in the direction of the increasing gradient of adhesion factors, the speed of growth and final length of axons are not only depending on the slope of such gradients, but also on the absolute range of spatial concentration of the adhesive molecules (fig. 1.11). These results suggest that the integration of surface cues by growth cone scanning is not only space dependent, but also cumulative over time [45].

Like 2D micro-patterned surfaces, structured environments in 3D are also suitable to direct neurites outgrowth. A study from Gomez et al. [46], suggested that neurons prefer growing on structured surfaces rather than planar patterned surfaces. Embryonic rat hippocampal cells were seeded at the junction between uniformly coated surfaces, and structured surfaces with adhesive grooves (1 – 2 μm width, depth and spatial period). Under these conditions around 70% of the axons of the positioned neurons are growing on the rough surface, choosing physical over chemical guidance.

Bédurier et al. [47], studied the growth of neurons on grooved surfaces (25 μm depth

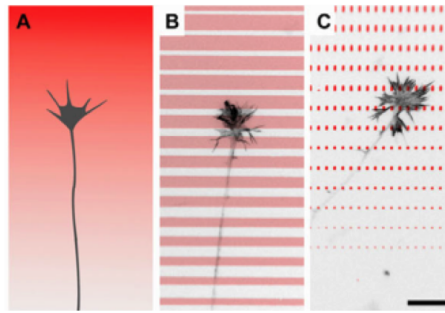


Figure 1.11: Substrate-bound gradients fabricated by microcontact printing. (A) Schematic of a growth cone in a continuous gradient. (B,C) Growth cones in different discontinuous gradients fabricated by microcontact printing: (B) a steep gradient built by an array of stripes and (C) a shallow gradient built by dots. Antibody-stained ephrin is shown in red; phalloidin-stained actin in black. Scale bar = 15 μm . Source: Von Philipsborn et al. [45].

and 5 – 20 μm width). They showed that the number of neurites as well as neurites length depend on the width and period of the pattern. In both cases, the largest values show the best efficiency. On the contrary, alignment of neurites with the main orientation of the grooves is increasing when geometrical parameters get smaller. This alignment is strongly dependant on the cell density of the culture, emphasizing the fact that cell-to-cell interactions are favoured over cell-to-substrate interactions. Similar studies of neurons growth on top of grooved surfaces, have been published [48–52]. Li and Folch characterized the maximum height that neurons can climb and estimated it around 15 μm [48] (fig. 1.12). Tuft et al. [49] investigated more the difference between several neuronal subtypes and glial cells. Francisco et al. discovered that chicken dorsal root ganglia neurons prefer wide ($>90^\circ$) over sharp ($<90^\circ$) turn [51].

Li and Folch [48], studied the role of the growth cone navigating on structured surfaces. When the growth cone is encountering an obstacle, it retracts and search for the widest open space accessible by changing the least its current orientation. Jang et al. [53], went even further in this explanation. They observed that the alignment of dendrites with 3D topographies is triggered by the interplay of two population of filopodia at the growth cone. The first population, perpendicular to the main orientation of the pattern, scans the surface back and forth and is highly unstable. The second population, at the tip of the growth cone, align with the substrate. This second population is stabilized by F-actin and drives axonal outgrowth. On flat substrates, only unstable filopodia are observed, causing neurite collapse from times to times. Consequently, neurite outgrowth is slower on flat surfaces compared to structured surfaces.

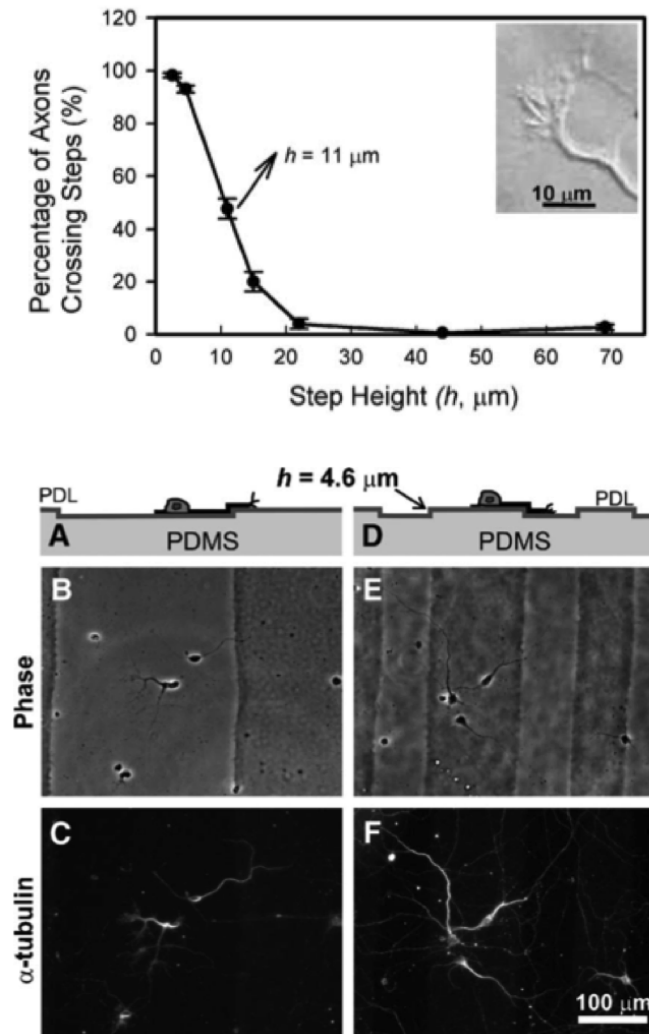


Figure 1.12: The top graph shows the percentage of axons that cross a step as a function of step height h . The graph shows that the percentage of axons that disregard the microtopography increases as the groove depth decreases, with a threshold at $11 \mu\text{m}$. Axons disregard shallow steps. Cross-sectional schematic representation (top row), phase-contrast (middle row) and corresponding α -tubulin immunofluorescence micrographs (bottom row) illustrating typical neurons cultured on $4.6\text{-}\mu\text{m}$ -deep, PDL-coated PDMS grooves. Note that neurons (both on the bottom surface of the grooves (A, B, C) and on the plateau surfaces (D, E, F)) ignored the topographical steps, extending axons freely into and out of the grooves. *Source: Li and Folch [48].*

In the specific case of neurons there is a major interest in guiding their axon, differentially from their dendrites, to be able to build oriented synaptic connections *in vitro*. Yet few studies have made the difference between axons and dendrites behaviours.

The study of Roth et al. emphasized the crucial role of mechanical tensions of the cytoskeleton in axonal outgrowth [43]. This study presents an elegant method to apply 2D micro-patterning techniques to build oriented neuronal networks *in vitro* that are controlled at the single cell level. If a neuron cell body adhere on a circular poly-lysine spot linked to a straight poly-lysine line and two sinusoid poly-lysine curves, then the axon will preferentially grow on the straight line, allowing it to maximise its tension (fig. 1.13). Using this strong polarization effect one can design networks of sinusoid and straight poly-lysine curves, with some nodes to place neuronal cell body. This way one can build single-neuron network with a fully controlled and oriented map of synaptic connections.

Building Neuronal Networks *In Vitro*

With the development of the micro-patterning techniques described above, neuronal networks started to be reconstructed and studied *in vitro* [54,55]. Most of these networks were controlled at the single cell level, with either reciprocal [56,57] or oriented synaptic connections [58,59].

I will now focus on another kind of *in vitro* neuronal networks that control synaptic connections at the population level. This kind of networks should recapitulate more the physiological conditions. However, we will see in chapter 2 that this control at the population level have some limitations. To build complex neuronal networks *in vitro* the combination of macro- and micro-patterns is required.

Compartmentalized neuronal cultures

In 2005, Taylor et al. [60] proposed a new microfluidic paradigm in which different neuronal populations are cultured in individual macrochambers (100 μm height), connected by thin micro-channels (10 μm height and width, diverse length) in which axons can grow but soma cannot enter (fig. 1.14).

This design was extensively used to study neuronal development, axonal myelination or axonal degeneration [61–63].

The design of Taylor et al. was a first step towards the reconstruction of neuronal networks *in vitro*. It allows to organize and segregate different sub-populations of neurons in different spaces. It also permits to guide axonal outgrowth and to form

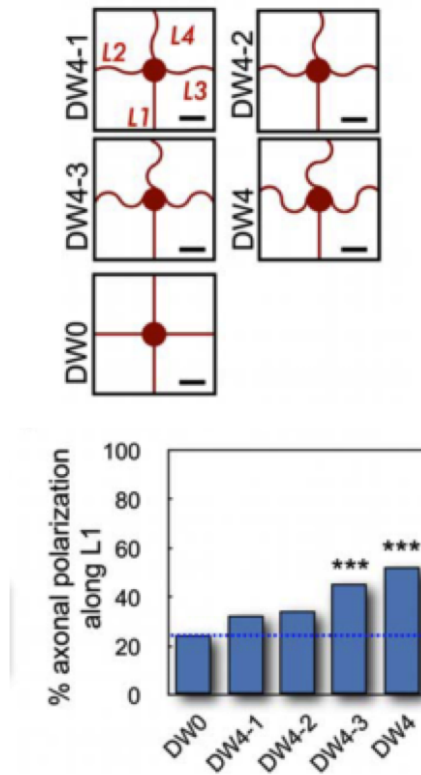


Figure 1.13: Influence of neurite curvature on axonal polarization. Top part: set of patterns of increasing curvature along directions L2, L3, L4; Scale bars = 20 μm . Bottom graph: Preferential axonal specification along the straight direction L1 were plotted from stage 3 neurons plated over DW0 (24.4%), DW4-1 (32.6%), DW4-2 (34.7%), DW4-3 (45.5%), and DW4 (52.3%) patterns. *Source: Roth et al. [43].*

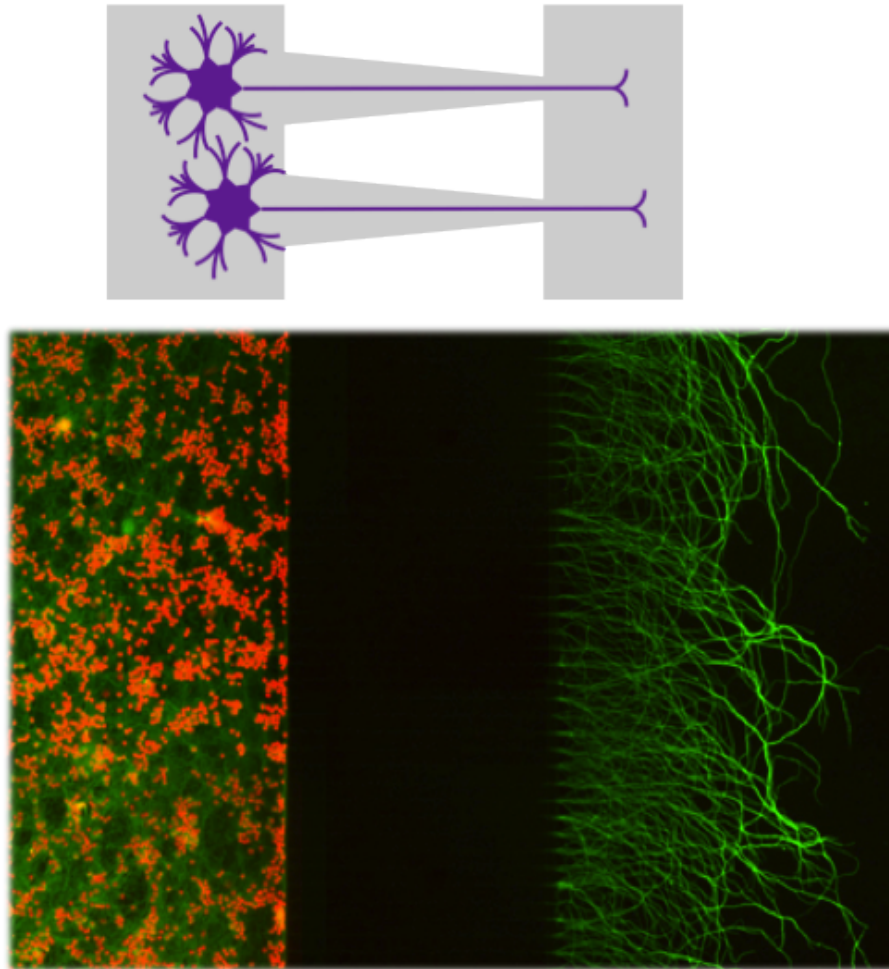


Figure 1.14: Axons (stained in green) are isolated from cell bodies and dendrites (nuclei stained in red). The array of micro-channels of $500\text{ }\mu\text{m}$ length and $10\text{ }\mu\text{m}$ width and height blocks the cell bodies in the left macro-channel but allows axons to cross to reach the right macro-channel. The axons growing in the micro-channels are not visible because they are not stained, probably due to antibodies absorption on the surfaces. *Credit for the fluorescent image: Sebastien Magnifico and Jean-Michel Peyrin.*

non-oriented synapses between two populations of neurons [64]. The next step was then to control the orientation of synaptic connections.

Oriented Neuronal Networks In Vitro

At MicroTAS 2008, Jean-Louis Viovy's and Bernard Brugg's teams first introduced the concept of "axon-diodes", imposing an oriented outgrowth to axons, and thus improving the physiological relevance of reconstructed networks. "Axon-diodes" are funnel-like micro-channels, with a wide "inlet" facilitating the entry of axons, and a narrow "outlet" preventing the entrance of axons, but yet allowing the exit of axons growing into the micro-channels [65] (fig. 1.15).

Other methods have been developed to control the orientation of synaptic connections *in vitro* [66–69]. The method promoted by Pautot et al. [66] used self-assembling nano-spheres coated with different antibodies to capture and segregate different cell populations. Other studies embedded neurons in gels and let them build synaptic connections, either randomly [68] or controlled by photothermal gel etching [69]. Finally the method used by Feinerman et al. [67] used the same funnel-shaped geometry to control connections polarity but in this case the "axon-diodes" were encoded by micro-contact printing instead of micro-channels.

The advantages and limitations of the "axon-diode" design will be discussed in more details in the following chapters but it appears to be a simple and robust design to build oriented neuronal networks that connects large populations of neurons. These networks are more relevant to mimic physiological pathways development or degeneration (refer to chapter 2). However the principal limitation of these networks is that many synaptic connections are not controlled. That can lead to non-physiological connections for complex neuronal networks comprising more than two population of neurons. More elaborate micro-patterning techniques can be developed to complement the role of "axon-diodes" and insure a better control of synaptic connections (refer to chapter 3). Finally, the reconstruction of neuronal networks *in vitro* really becomes powerful if we find also means to interact with these networks and investigate neuronal networks dynamics. A novel method allowing these interactions will be presented in chapter 4.

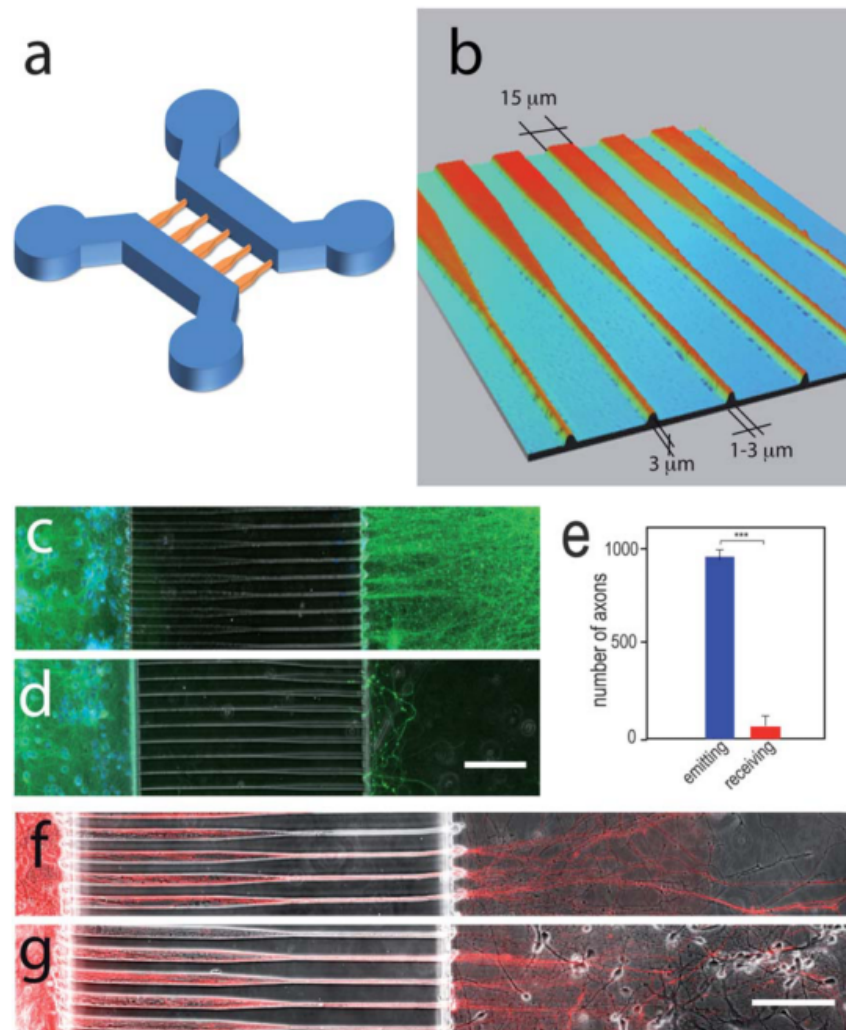


Figure 1.15: In vitro reconstruction of a neuronal network using “axonal diodes” in microfluidic culture devices. (a) Global 3D view of the microfluidic device comprising asymmetrical microchannels (b) 3D view of the funnel-shaped micro-channels as observed by white-light optical profiling (WLOP) with an interference microscope. Immunofluorescent images of microfluidic cultures (green: α -tubulin, blue: Hoechst) in which cortical neurons were seeded either on the wide (c, 15 μm) or the narrow (d, 3 μm) side, at a concentration of $45 \cdot 10^6$ cells per ml. (e) Quantification showing that the axonal diodes efficiently polarize axonal growth (***) p-value < 0.001). Phase contrast image combined with epifluorescence of a reconstructed polarized neuronal-network after 8 days in vitro. To visualize individual axons of cortical neurons, cells were transduced with a m-cherry Sindbis viral vector (red). Cortical neurons projecting their axons into the second chamber without a target (f) or in the presence of striatal neurons seeded in the second chamber (g). Note that striatal neurons are not infected by the virus, indicating an effective compartmentalization of the two culture chambers. Scale bar 50 μm . Source: Peyrin et al. [65].

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Chapter 2

Building Oriented Neuronal Networks In Vitro

2.1 Background and challenges

During the first phase of nervous system development, apoptosis ensures proper neuronal connectivity by eliminating around 50 % of cells. Then, during the second phase, axonal pruning and synaptic modelling ensures even more accurate connectivity. During adulthood, synaptic remodelling is the main mechanism for the plasticity of neuronal networks. These mechanisms are deregulated in most neurodegenerative diseases [1]. In Alzheimer's disease (AD), recent studies reveal that specific subsets of neurons show early signs of synaptic loss and axonal degeneration leading to neuronal network dysfunction long before neuronal death occurs. The early phase of nerve terminal disorder is supposed to be responsible for cognitive decline and memory loss observed in AD patients [2–4].

AD brain lesions consist of two main hallmarks responsible for neuronal dysfunction: i) extracellular deposits of beta amyloid peptides tangled in oligomers or amyloid fibrils ($A\beta$ plaques), and ii) intracellular anomalies like the accumulation of $A\beta$ and the hyper-phosphorylated tau protein, which forms paired helical filaments [5–7]. Neurodegenerative diseases like AD proceed over several years in humans, though the clinical symptoms are only detected in the late phase. Despite the fact that the disease develops slowly at the macroscopic level, the active phase of apoptosis occurs quickly ($\leq 20h$) at the cellular level. The relationship between

these two levels is still not understood.

Importantly, AD related degenerative processes progress spatially and temporally during the disease, the cortico-hippocampal pathway (fig. 2.1) is the first area to degenerate [8,9]. The reasons why AD related neuro-pathological hallmarks progress are not known yet. Neuronal activity, and thus connectivity, plays an important role in neuronal survival. Therefore, progressive synaptic and axonal degeneration in AD may accelerate the adverse effects of amyloids and trigger a vicious circle leading to neuronal death.

In the context of AD, when a subset of neurons in a given network is damaged by amyloids, it is important to understand the relationships between the single neuron level and the neuronal network level. Classic experimental models to study AD range from whole animal models, which preserve the anatomical structures, to dissociated cell culture systems, which allow detailed manipulation of cell phenotype. However, both models have significant limitations: either they greatly limit experimentation at cellular and sub-cellular level or lack the highly ordered and instructive brain environment (fig. 1.4, pp 13).

New *in vitro* experimental models were needed to facilitate both individual cell manipulation and brain connectivity reconstruction. Due to close collaboration between the Jean-Louis Viovy team and the Bernard Brugg team, a new microfluidic cell culture device was developed allowing the reconstruction of oriented neural networks [10]. This device was inspired by the microfluidic chips designed by Noo-Li Jeon in 2005 [11]. Basic microfabrication and cell culture techniques were developed and the first two-compartmented (2C) oriented network with cortex neurons (Cx) connecting striatum neurons (St) was built. This first network was then optimized, fully characterized and extensively used for neuro-degenerative studies in the Bernard Brugg team.

The basic design of our devices comprises two large channels that allow neuronal seeding and development. These two culture chambers are separated by an array of narrowing micro-channels, called “axon-diodes”, that allow axons to grow in one direction but block them in the opposite direction.

During my PhD, I developed hippocampal cultures and built the second 2C oriented neuronal network with Cx connecting neurons from the hippocampus (Hi). The reconstruction of this novel network had the double interest of generalizing the techniques and offering a new platform for neuro-degenerative studies. Cx-Hi networks are well suited to investigate degenerative processes occurring in Alzheimer’s Disease (AD). Histological and *in vivo* animal studies suggest that AD begins in the hippocampus and then propagates in the whole cortex [8,9,12]. We question if this

propagation follows neuronal networks connectivity. Convergent results seem to be in favour of this hypothesis but a formal experimental proof is still lacking [12–15]. *In vitro* oriented neuronal networks could be used to test this hypothesis.

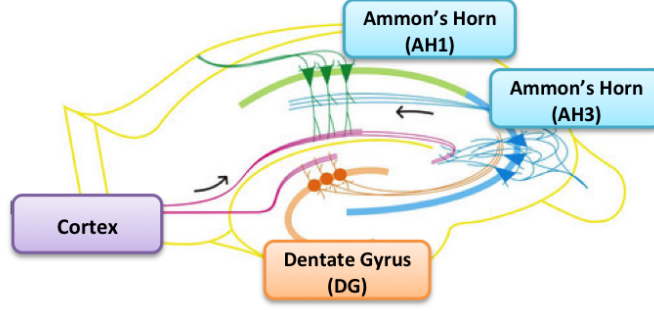


Figure 2.1: Scheme of the mouse perforant path with the main cortex/hippocampus synaptic connections *Image modified from Toshi Nakashiba, MIT.*

The next challenge was to build a more complex network mimicking the perforant path. The perforant path comprises three neuronal populations: cortex (from entorhinal cortex), dentate gyrus (DG, from the hippocampus) and Ammon's horn (AH, from the hippocampus), connected sequentially. Cortex are connected to dentate gyrus, which are connected to Ammon's horn (fig. 2.1). Building an oriented network with three neuronal populations connected sequentially was a good way to push forward the 2C neuronal network design.

This perforant path can be used to study the propagation of AD lesions through oriented synaptic connections *in vitro*, mimicking the early stages of AD.

2.2 Materials and Methods

2.2.1 Design and Micro-fabrication

Basic design of our chips

The simplest microfluidic design used for neuronal networks reconstruction comprises two parallel channels ($55\text{ }\mu\text{m}$ height $\times$ 1 mm width $\times$ 4 mm length) separated by a perpendicular array of nearly one hundred micro-channels ($3\text{ }\mu\text{m}$ height $\times$ $500\text{ }\mu\text{m}$ length $\times$ $3 - 15\text{ }\mu\text{m}$ width) (fig. 2.2).

The two biggest parallel channels are used to seed the different populations of neurons. I will call these channels “macro-channels” or “culture chambers”. The

smallest channels, so-called “micro-channels”, are too thin for the neuronal cell bodies to crawl in, however they allow neurites to cross from one chamber to the other.

The size of the micro-channels allows both axon and dendrite guidance. However, the micro-channels are long enough to allow crossing by axons only. This way the synaptic connections between the two populations are formed in the chambers and are more concentrated close to the micro-channels.

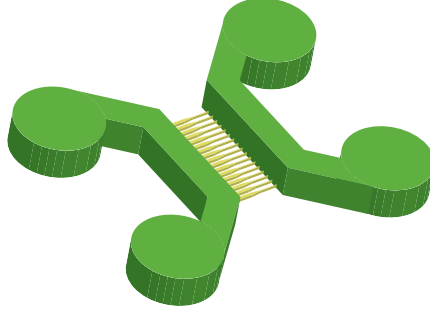


Figure 2.2: 3D illustration of the basic design of our microfluidic chips for neuronal networks reconstruction (*not to scale*).

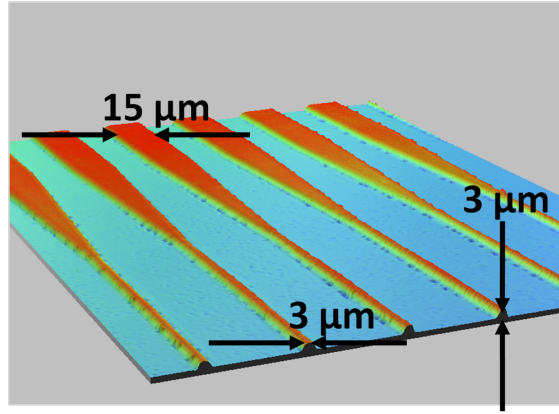


Figure 2.3: 3D reconstruction of the axon-diodes realized with a 3D optical profilometer.

If the micro-channels are straight, axons can cross in both directions leading to symmetrical synaptic connections between the two populations. To build oriented synaptic connections, i.e. asymmetrical connections with only one population sending axons towards the other, the “axon-diodes” are used (fig. 2.3). Following probability laws, their funnel-like shape facilitate the entrance of axons at their wide

side and almost completely prevent neurites to enter at their narrow side.

Throughout my PhD manuscript I call the population that sends axons and builds pre-synaptic buttons on the dendrites of the population in the opposite chamber the “input-population”. The other population, which receives axons from the input-population and builds post-synaptic buttons, is called the “output-population”.

Fabrication of the masters by photo-lithography

In photo-lithography, a surface is uniformly coated with a resist that is sensitive to UV light. Under UV illumination through a mask, the resist is either destroyed (positive resist) or polymerized (negative resist) selectively in the transparent regions of the mask. After this illumination, a development step allows us to rinse the destroyed or non-polymerized areas of the resist.

During my PhD, the masks were drawn using the computer assisted drawing software QCAD (Ribbonsoft, Inc., USA). The masks were all fabricated by the Swiss company Selba (Selba, Inc., Switzerland). The masks for the micro-channels were made by chrome deposition on a 3 mm quartz slide. In lithography processes quartz is used because it is transparent to UV light. The masks for the macro-channels are printed with a resolution of 56000 dpi on plastic films.

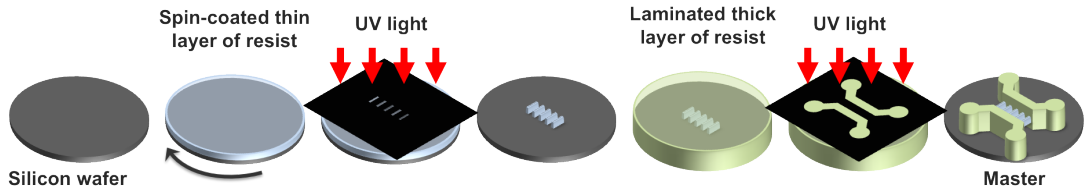


Figure 2.4: Scheme of the lithography steps for the fabrication of the masters (*not to scale*).

The fabrication of the masters (or molds) is realized in a clean room. The supporting materials for the masters are usually silicon wafers ($\varnothing = 5$ cm) and occasionally 1 mm thick glass slides. Two steps of lithography are required to build structures with two different heights ($55\text{ }\mu\text{m}$ and $3\text{ }\mu\text{m}$) (fig. 2.4). The $3\text{ }\mu\text{m}$ high micro-channels are created first, by using the negative photo-resist SU8-2002. This resist is spin-coated at a given speed controlling its height. The correspondence between the speed of the spin-coating and the height of SU8 resists is well-characterized. Nevertheless the viscosity of these resists increases by solvent evaporation when the resist age in bottles. For this reason, the resist height need to be checked regularly and the speed of the spin-coating adjusted to obtain the desired height.

The macro-channels are fabricated by using the negative photo-resist SY 355, laminated on top of the thinnest resist after its development. The resist SY 355 consist in a dry film of 55 μm height protected on each side by thin plastic films. The tricky parts of the lamination protocol are to avoid bubble formation between the wafer and the resist, and to prevent wafer breaking. However, the thickness of dry films are better controlled than the thickness of spin-coated resist.

The surface of the laminated resist is much rougher than the surface of the SU8 resist. This has no real consequences for our applications, but could trigger some perturbations in the laminar flow in the channels.

The masters prepared by lithography can be copied with a polyester resin. These replicas are much more robust and last longer. One silicon master can be used to make 10 to 30 resin copies and one replica can serve as a mold for up to 100 PDMS chips. After this number, the resin micro-structures are smoothed down, the micro-channels clogged and block axonal outgrowth, the replica need to be replaced. One master can serve to make up to 2000 chips on average. This large number need to be put in relation with the large number of chips needed for biological experiments (around 30 to 100 chips per experiment i.e. 30 to 100 chips per week). Thus the masters need to be renewed at least every three months.

Fabrication of the chips by soft-lithography

PDMS was used to make all my chips. The base of PDMS was mixed with its curing agent in 10:1 w/w proportions. Then the mix is vigorously agitated, degazed under vacuum and poured on a master. After 2-3 hours of curing in an oven at 65°C, the PDMS can be peeled off from the mold. 3-4 mm holes are then punched in the PDMS to form the inlets and outlets of the two parallel macro-channels. These wells are large because most of the fluid supplies are done by classic pipetting.

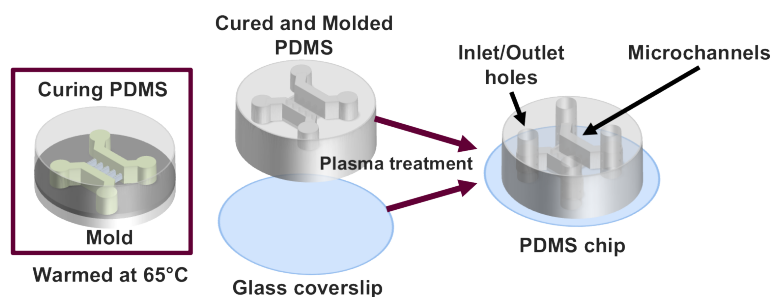


Figure 2.5: Scheme of the soft-lithography steps for the fabrication of the chips (*not to scale*).

Then the PDMS and a glass coverslip are washed with isopropanol and dried out by compressed air. The structure surface of the PDMS and one of the glass surface are activated by air plasma for 30 to 60 seconds and irreversibly bonded together by manual pressure to form the microfluidic channels. The plasma treatment turns the PDMS surface to be hydrophilic and thus allows the filling of the channels with aqueous solutions such as PBS (phosphate buffer saline).

Prior to neuronal seeding the chips are illuminated with short-wavelength UV light for 10 minutes to ensure their sterility. Finally the inner surfaces of the channels are coated with poly-D-lysine overnight at 37°C to allow neuronal adhesion to the substrates.

2.2.2 Neuronal primary cultures

Neurons from different parts of the brain can be grown in culture for several days or weeks depending on the protocols. Primary neuronal cultures are prepared by dissecting differentiated but non-developed neurons from embryonic brains. After the dissection and dissociation of embryonic tissues, neurons are grown *in vitro*. Neurons are taken together with glial cells that will grow and proliferate in the cultures. To culture a given type of neurons, a specific area of the brain needs to be isolated at a specific embryonic stage close to its neurogenesis peak (i.e. during neuronal proliferation, after neuronal differentiation and before neuronal maturation).

Primary culture of cortex

The cortex is taken off from the two hemispheres of mice embryos brains between E14 and E16 (wild-type strain) (fig. 2.7). Tissues are dissected on ice under a binocular microscope. The tissues are then incubated with an enzyme (trypsin or papain) that digests the extra-cellular matrix. This digestion step is followed by mechanical trituration (by pipetting) to individualize cells in suspension. The DNA released by damaged cells needs to be then digested because it would cover the poly-D-lysine and prevent neuronal adhesion to the substrate during seeding.

Dissecting 12 embryos gives around $50 \cdot 10^6$ cells. After two centrifugations, the cells are suspended in culture medium at an average concentration of $40 \cdot 10^6$ cells/mL. 2 to 3 μ L of this solution is then injected in the inlet of one macro-channel. The cells flow in the chamber and progressively attach to the coated surfaces while the speed of the flow decreases until reaching equilibrium. After a few minutes, media is flown in the chambers and the cultures are placed in a humid incubator at 37°C and 5% CO₂.

Just after seeding neurons have a spherical shape. They develop their neurites in the chip and axons can cross the micro-channels in 4 to 6 days depending on where the cell body they belong to is positioned. To my experience cortical cultures can be kept up to 6 weeks in our microfluidic systems. However, I usually only grew them for 15 to 17 days as it was sufficient for my experiments. Cultures are easier to handle at young ages. The culture medium need to be changed every 4 days approximately but this time becomes less critical while the culture is ageing. The medium consists in a buffer solution of DMEM with glutamax, supplemented with 10% foetal bovine serum, one anti-oxidative molecule (B-27), one growth factor molecule (N-2) and antibiotics (penicillin and streptomycin).

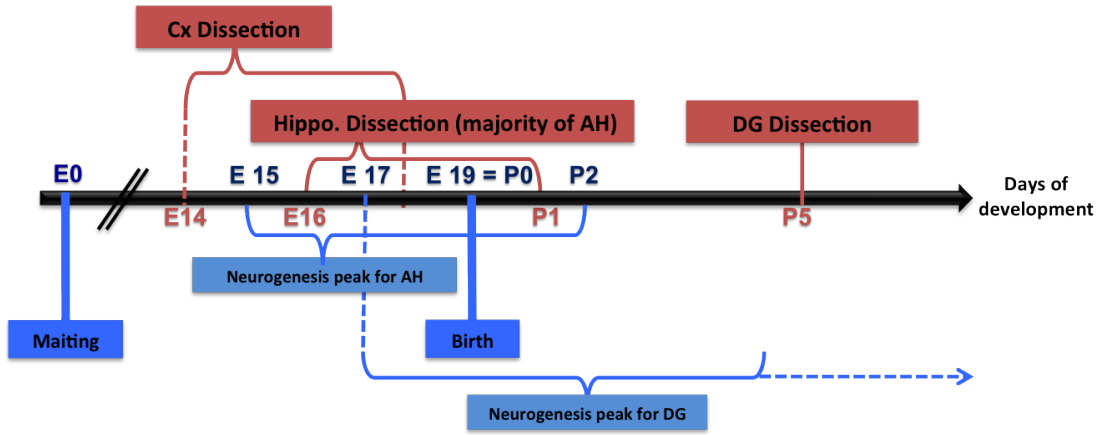


Figure 2.6: Approximate timeline of the proliferation peaks for different neuronal populations and corresponding usual days of dissection.

Primary culture of hippocampus

The hippocampus is named after its shape, a spiral shape that looks like the tail of the marine hippocampus. The hippocampus is constituted by two different neuronal subtypes, the Ammon's horn and the dentate gyrus neurons, which form two parallel and partly interlocked layers tangential to the spiral shape (fig. 2.1). During mouse development the Ammon's horn neurons proliferate first between 15 and 19 embryonic days. The dentate gyrus proliferate during the first 10 days after birth. As a consequence, if you dissect the whole hippocampus between E16 and P1, you will have a mix of AH and DG with a majority of AH. If you dissect pups around P5 and mechanically separate DG from AH, DG neurons can be cultured at high purity.

The protocol for the dissection of the hippocampus or hippocampus subparts are basically the same as the one for the cortex. Older tissues are more difficult to digest so the enzymatic digestion time is usually longer for hippocampus tissues. This difficulty of digestion also implies that hippocampus neurons are more

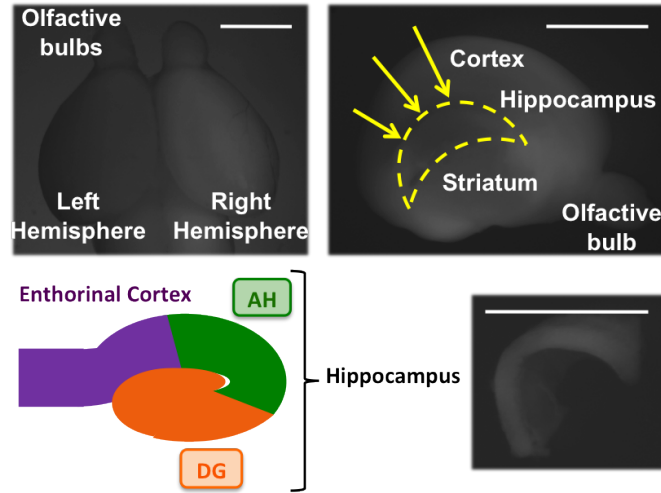


Figure 2.7: Translight pictures of dissected mice brain, hemisphere and hippocampus; and simplified scheme of the perforant path *Scale bar 1 mm*.

likely to aggregate when conserved in solution at high concentrations. As a consequence, hippocampus cells suspensions need to be regularly agitated during seeding.

The hippocampus is much smaller than the cortex. After dissection and dissociation of the whole hippocampus of 12 embryos, the number of cells harvested is typically around $4 \cdot 10^6$. For DG tissues the number of cells can go down to $2 \cdot 10^6$. Therefore hippocampal neurons are generally seeded at a lower concentration than cortical neurons, around $20 \cdot 10^6$ cells/mL.

The culture medium used for all hippocampal and cortical neurons is the one describe above for Cx. This might not be ideal for hippocampal neurons but it is much more practical for handling the fluids in the chips.

2.2.3 Imaging methods

Two kinds of imaging were done on the neuronal cultures, “dead” imaging (fixed and immunostained neurons) or live imaging (calcium imaging).

Fixation and immunostaining

The cultures can be fixed at any time by flowing paraformaldehyde (PFA 0,4%) in the channels and incubating it at room temperature during 10 minutes. The PFA is penetrating the cell membrane and the alcohol groups of the PFA mainly react with the acid carboxylic groups of the proteins and nucleic acids inside the cells to form esters. Within a couple of minutes the cells die due to molecular cross-linking and immobilization. After this treatment the tissues and cells are stable and can be

conserved in sterile aqueous solutions for several weeks.

After fixation, the cells can be colored by an immunocytochemistry method. The principle of this method relies on the specific recognition of a protein of interest by its corresponding antibody. The fixed cells are treated by a detergent (Triton X-100 0,2%) to open pores in the membranes and make the inner parts of the cells accessible. A solution containing antibodies directed against a protein of interest is then incubated with the cells. This antibody can either be directly coupled with a fluorophor or recognized by a secondary and more generic antibody coupled with a fluorophor. Three to four proteins can usually be highlighted by this method, using antibodies coupled with fluorescent molecules that emit in red, green, blue and eventually far-red light. Throughout my PhD, images has been taken with epi-fluorescent inverted microscopes (Nikon or Zeiss).

Different kinds of immunostaining have been performed: i) cytoskeleton proteins staining to observe the shape and morphology of the neurons (β -III tubulin to stain the whole neurons and MAP-2, which stands for microtubule associated protein 2, to specifically stain dendrites), and ii) phenotypic protein staining to characterize the percentage of different populations of cells (Prox-1 to stain specifically DG neurons and GFAP, for Glial fibrillary acidic protein, to stain specifically glial cells).

Live calcium imaging

Calcium imaging is a method that allow to image live cells. It can in principle be done at any age of the culture but as it is reflecting the electrical activity of the neurons, it is usually done after 10 days of development *in vitro*.

The cells are incubated during half an hour with a buffered solution containing a fluorescent dye (Fluo-4 or Calcium Orange) that allows us to detect variations of calcium concentration. The fluorescent yield of such fluorescent dye increases if the dye binds with Ca^{2+} ion. The incubation solution is then rinsed and the neurons are incubated for another half hour in their regular medium. The neurons are then observed with an epifluorescent microscope and fluorescent intensity changes in their cytoplasm but most visibly in their cellular bodies. These waves of fluorescent intensity reflect waves of calcium concentration in the cytoplasm of the neurons, which reflect the electrical activity of the neurons. It is admitted that a calcium peak corresponds to an action potential sequence, also called spike train.

The different steps of a typical experiment are summarized in the figure 2.8. The minimal maturation time of the neuronal culture for an immunostaining experiment is about 5-10 days (depending if cytoskeletal or phenotypic proteins need to be

observed) and the typical time for an activity recording experiment is around 3 weeks (imaging steps included).

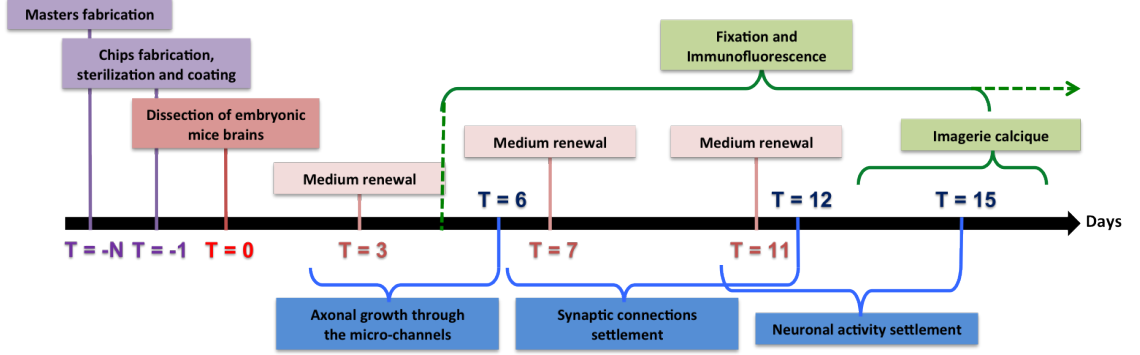


Figure 2.8: Typical experimental timeline.

2.3 Results and Discussions

2.3.1 Two-compartmentalized and oriented cortico-hippocampal networks

Purity of the cultures

The perforant path is a neuron circuit comprising three populations of neurons connected in series. To move towards a tri-partitioned network mimicking the perforant path, it is necessary to separate and maintain separately in culture DG and AH neurons.

To perform this separation, we first wanted to use the method proposed by Baranes et al. [16]. This method, developed in rats, allows to separate DG from CA by using their density difference due to their difference in size ($\varnothing \approx 30 \mu\text{m}$ for AH against only $10 \mu\text{m}$ for DG). It consists in collecting the entire hippocampus of rat pups from P1 to P5, and then separate the two cell types using a discontinuous Percoll gradient. This protocol failed to separate our hippocampal neurons of mice because the number of cells collected was too low; the cells were trapped in the Percoll and could not be recovered. A second option was to take the whole hippocampus, slice it, and then separate the DG from AH, helped by the special spiral morphology of the hippocampus, on each slices [17]. This protocol was tried once and abandoned because it was too tedious and time consuming.

A third option was chosen, which takes advantage of the late appearance of the structure of the DG during SNC development. Indeed, the first neurons DGs appear

at the end of the embryonic period (E18), and proliferate to form at P5 a structure well differentiated from AH. Thus, if the hippocampus is taken from embryos around E16, mostly AH neurons are harvested. At P5 when the two structures are clearly differentiated, they can be mechanically separated to collect only DG neurons. At this stage of development DG do not yet have neurites, while AH have stopped proliferating and already started developing their neuronal extensions. Thus, even if the micro-dissection is not perfect, AH contaminants may not survive the dissociation step.

The purity of the cultures were determined by phenotypic immunostaining. If the whole hippocampus from P0/P1 pups are dissected the culture is composed of approximately 70 % of AH and 30 % of DG. The purity of AH cultures, after dissection of the whole hippocampus of E16-E18 embryos, was around 80 %, while the purity of DG cultures, after dissection of pups at P5, was around 95 %.

The purities of our cultures, measured by a phenotypic staining of the transcription factor Prox-1 specific of DG, are comparable to those obtained by Percoll sorting [16] and micro-dissection of slices [17]. The morphology of the two isolated populations is also consistent with literature [18, 19]. AH neurons exhibit a large pyramidal soma of about 25 – 30 μm in diameter and a complex dendritic tree. DG neurons have a round cell body round of about 10 – 15 μm in diameter, and a usually simpler dendritic tree (fig. 2.9).

Despite good separation of the two populations of neurons and good purities of the cultures, a problem persists in cultures of DG. When neurons are collected at post natal stages, many glial cells are seeded in the chips. Unlike neurons, glial cells can proliferate, invade the culture chamber, and may disrupt the development of neurons. In DG cultures, there are about 20 % of glial cells that proliferate until reaching 60 % of the total population after ten days *in vitro* (fig. 2.10). Attempts to control this proliferation by the anti-mitotic factor Ara-C (cytosine arabinoside or cytarabine) were not conclusive. A concentration of 6 $\mu\text{mol L}^{-1}$ in Ara-C does not reduce significantly the proliferation of glial cells and reduce neuronal survival. Other strategies need to be explore to separate neurons from glial cells or to enrich the DG population prior to seeding.

Glial cells have an average diameter of 15 – 20 μm . They are larger than DG neurons that have a diameter of 10 μm . Microfluidic methods exist to separate particles or cells according to their size [20–22]. An hydrodynamic method was already used to separate neurons from glial cells based on their size [23, 24]. We could apply this method to separate DG from glial cells.

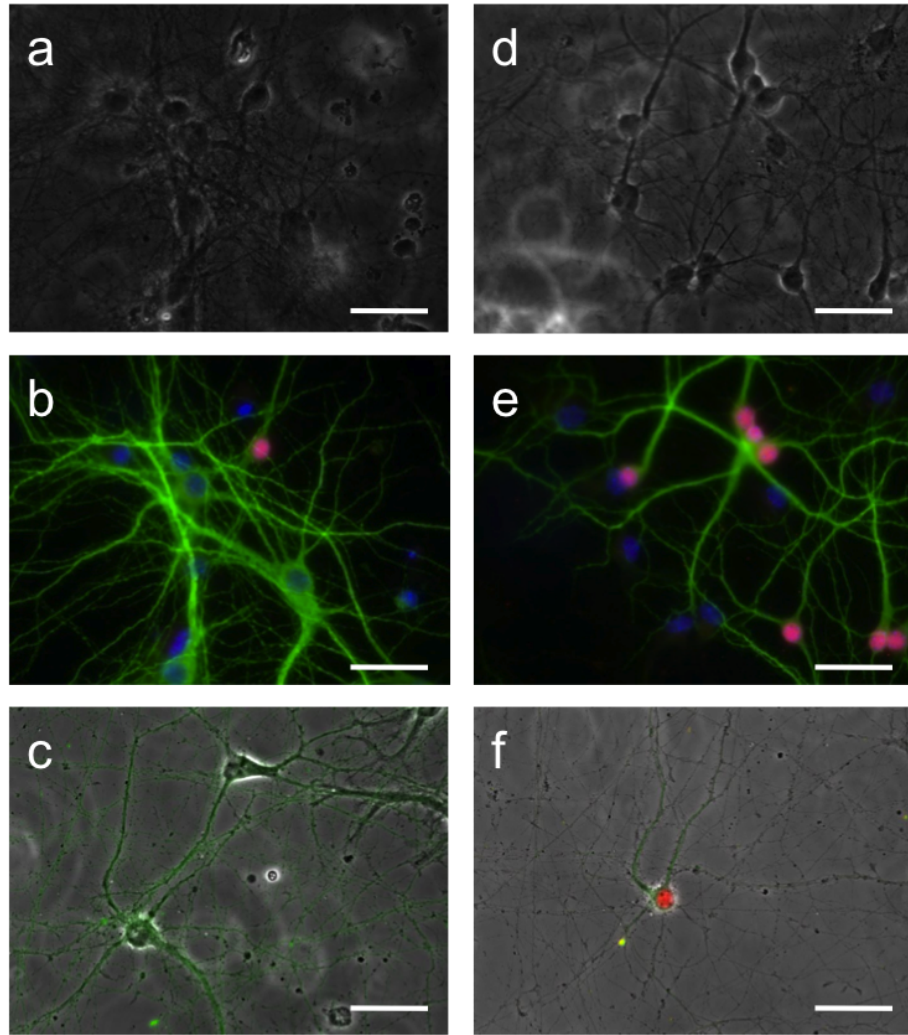


Figure 2.9: Purified cultures of AH (a, b, c). Purified cultures of DG (d, e, f). (a, d) translight images, (b, e) immunostaining of tubulin (in green) and Prox-1 (in red) that is specific to DG. (c, f) merge of translight, green and red images for isolated neurons. Magnification $\times 400$. Scale bars 40 μm

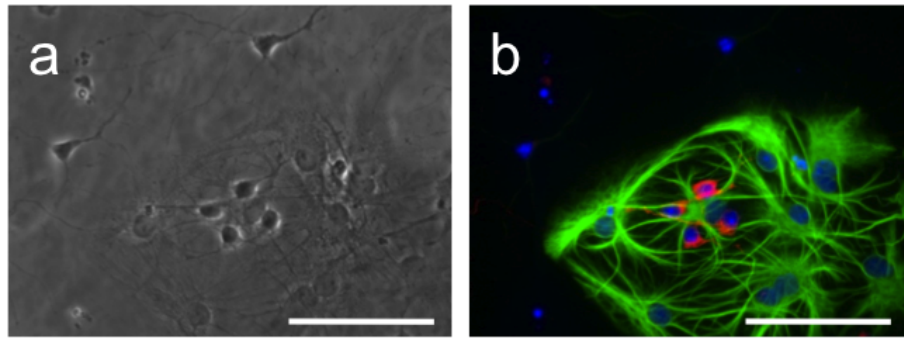


Figure 2.10: Proliferation of glial cells in DG cultures at DIV8. (a) *translight image*, (b) *immunostaining of GFAP (in green) that is specific to glial cells, and MAP-2 (in red) that stains the dendrites of the neurons*. Magnification $\times 400$. Scale bars $40\ \mu\text{m}$

Synaptic connections

Synaptic connections were observed by immunostaining in two-compartmented networks: Cx-DG and Cx-AH. Synapses formed on the dendritic tree and on cell bodies for both AH and DG.

It would have been interesting to study further this connections, evaluating if the synapses between cortex and hippocampus neurons (AH or DG) were synapses “en passant” (for which the axons continues towards next synapses, next dendrites or next neurons) or terminating synapses.

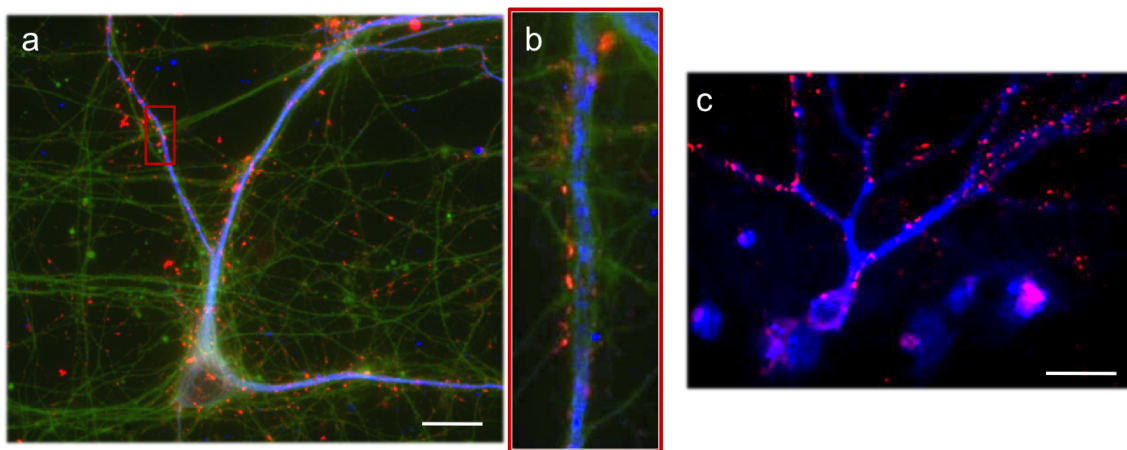


Figure 2.11: Synaptic connections between cortical fibers and AH (a,b) or DG (c) isolated neuron. *Immunostaining of α -tubulin (in green) synaptophysin (in red) and MAP-2 (in blue)*. (b) *is a magnification of the red box in image (a)*. Magnification $\times 400$. Scale bars $20\ \mu\text{m}$

The functionality of these synaptic connections were evaluated by calcium imaging. Activity of both cortex and hippocampus neurons were observed. However the direction in which neuronal signals propagate between these two populations was not yet tested. Cross-correlation analysis must be done to evaluate this direction, because cortex and hippocampus show spontaneous activity when cultured separately.

2.3.2 Towards mimicking the perforant path in vitro

First design and problems encountered

To build 3C networks, a simple design was first used that was just an extension of the 2C design. A third macro-channel parallel to the two first ones was added. This third culture chamber was separated from the second one by an array of “axon-diodes”.

This design was used to validate the sequential seeding of three different neuronal populations in the same microfluidic chip. Cortical and AH neurons were dissected and seeded simultaneously from E16 embryos on the first day and DG neurons were dissected and seeded the day after from P5 pups. Both dissections could not be realized on the same day because of time consideration. If the cells are seeded two days or three days in a row, they adhere nicely to the surfaces. Beyond three days after first population seeding, cells do not adhere any more, probably because the poly-D-lysine coating is covered by large proteins from the medium or cell secretions.

However this simple parallel design did not recapitulate the physiological synaptic connections topology of the perforant path. Three problems were raised:

- Cortical axons could cross the DG chamber and reach the AH chamber (fig. 2.13);
- DG neurons were too sparse in the medium chamber and most of the time did not manage to send axons across the micro-channels barrier;
- AH axons can cross micro-channels in the blocked direction of “axon-diodes” (fig. 2.14).

The selectivity of “axon-diodes” for AH axons was decreased to approximately 70-80 %, as compared to the selectivity of 97 % for cortical axons.

Strategies adopted and results

The strategies adopted to overcome these limitations were respectively:

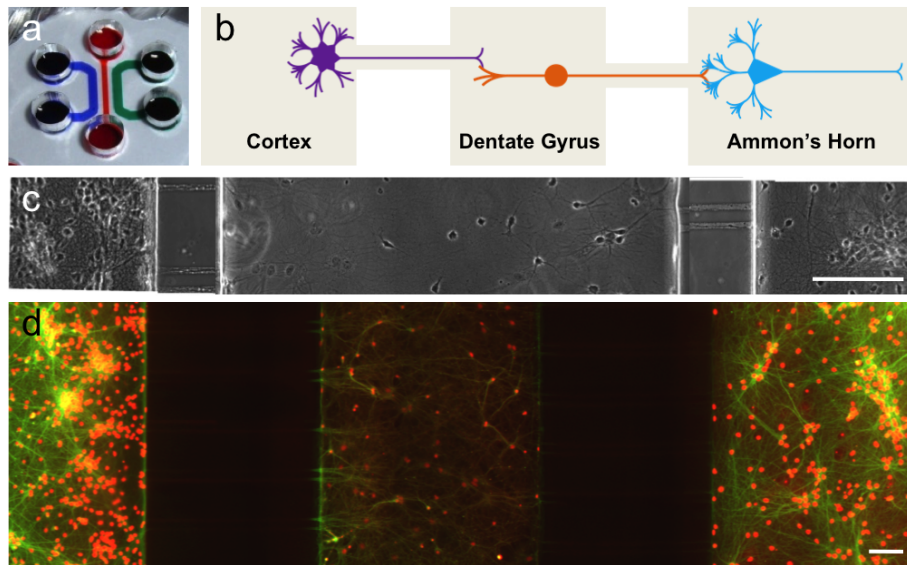


Figure 2.12: Parallel design for the reconstruction of the perforant path. (a) Picture of the whole circuit with the three parallel chambers filled with inks. (b) Schematic illustration of the network. (c) Translight overlaying pictures of the three chambers seeded with Cx, DG and AH neurons (from left to right). (d) Immunostaining of α -tubulin (in green) and Hoechst stained nuclei (in red, false color). Scale bars 100 μm

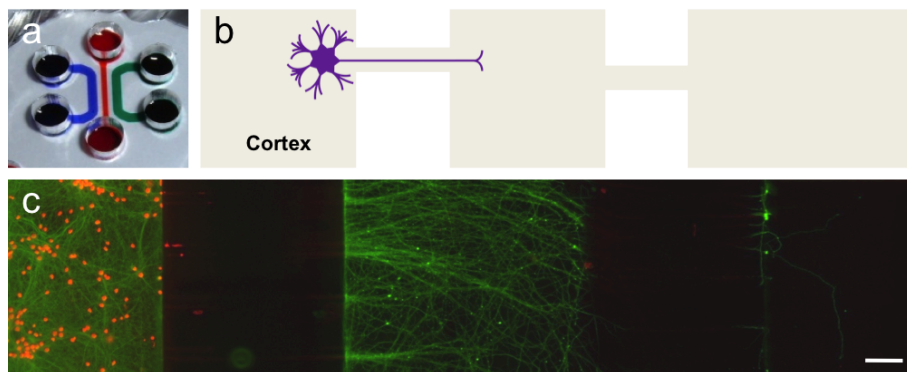


Figure 2.13: Cortical axons crossing towards the third chamber. (a) Picture of the whole circuit with the three parallel chambers filled with inks. (b) Schematic illustration of cortical seeding. (c) Immunostaining of α -tubulin (in green) and Hoechst stained nuclei (in red, false color). Scale bars 100 μm

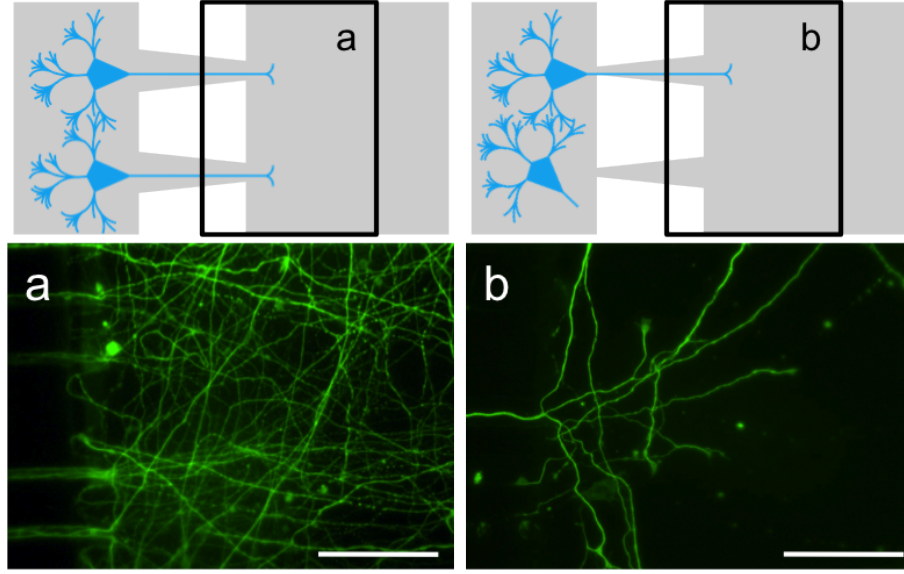


Figure 2.14: AH axons crossing micro-channels in the wrong direction of “axon-diodes”. *Top part: schematic illustration of the seeding vs pictures set-up. Bottom part: (a,b) Immunostaining of α -tubulin (in green). Scale bars 40 μ m*

- perpendicular architecture of the three macro-chambers;
- co-flow seeding for the DG population;
- seeding AH neurons at low densities.

The perpendicular architecture developed to prevent cortical axons over-shooting is illustrated in figure 2.15, and a picture of the chip with the three macro-channels filled with ink is shown in figure 2.16 d. This new design was first seeded with cortical neurons only in the first chamber (fig. 2.16 a). When they reached the second chamber, cortical axons bundles are de-fasciculating progressively while getting away from the micro-channels exit but few of them crossed the second and perpendicular serie of “axon-diodes” to reach the third chamber. The problem only remains for the micro-channels that are the closest to the first micro-channels exists because axons seem to have a tendency to follow corners or ridges. When Cx were seeded in the first chamber and DG in the second chamber, we could observe that cortical axons were constrained in the vicinity of micro-channels exists and DG axons crossed the second “axon-diodes” barrier (fig. 2.16 b). Finally, as a proof of concept, Cx, DG and AH neurons were seeded in the 3C perpendicular chip (fig. 2.16 c,e,f) and synaptic connections have been observed between the three population. However, the directionality and functionality of these connections should be tested more quantitatively.

Meanwhile, a chip design was developed to allow better placing of cell bodies in the macro-channels. Indeed, we can estimate that only the axons of cell bodies

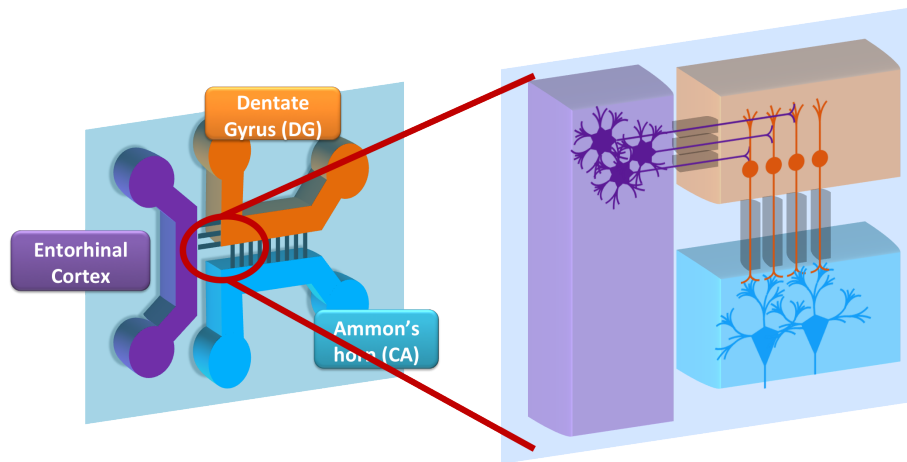


Figure 2.15: Scheme illustrating the 3C perpendicular network mimicking the perforant path. (*Not to scale*)

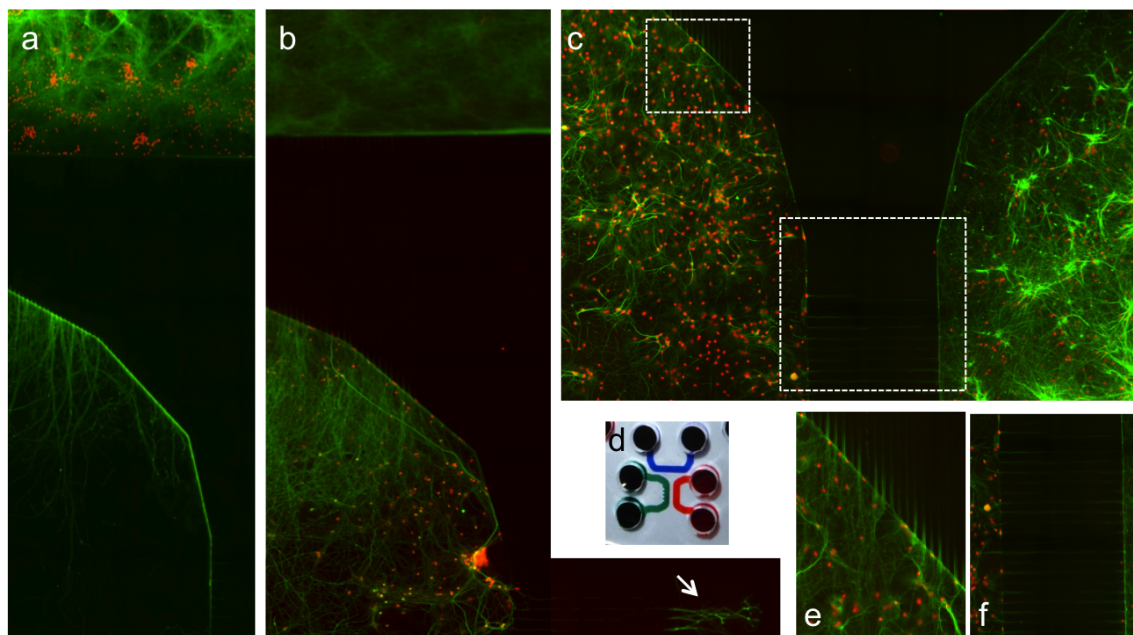


Figure 2.16: Reconstruction of the perforant path with a perpendicular design of the chips. (d) Picture of the whole circuit with the three parallel chambers filled with inks ; (a,b,c,e,f) immunostaining of α -tubulin (in green) and Hoechst stained nuclei (in red, false color). (a) Cortical neurons seeded alone in the first input chamber, top chamber on the picture ; (b) Cx and DG neurons seeded in the two first chambers, evidence of DG axonal crossing in the third chamber highlighted by a white arrow ; (c,e,f) DG and AH chambers view and magnifications in a chip seeded with the three types of neurons.

located in the vicinity of the micro-channels (i.e. not more than 500 μm away from them) can cross and reach the next chamber. A new design was developed using two seeding wells connected to two seeding channels (500 μm wide and same length) joining in the same macro-channel (1 mm wide) (fig. 2.17(a)). Knowing that flows in our microfluidic chips are laminar, two different solutions can then flow in parallel in the macro-channel without mixing. If cells in suspension are only introduced in one of the wells and the other only contain medium, cells are only seed in one half of the chamber (fig. 2.17(b,c)).

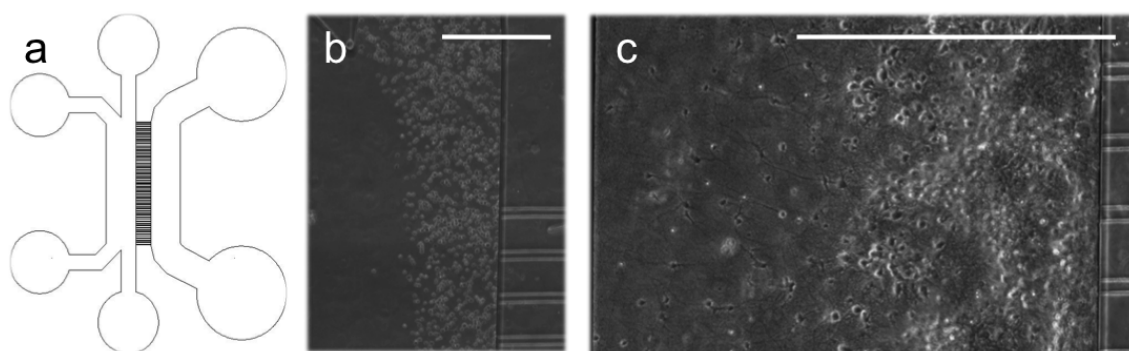


Figure 2.17: Co-flow seeding of DG neurons. (a) QCAD design ; (b) DIV0 translight picture taken right after seeding the DG neurons ; (c) DIV5 translight picture. Scale bars 500 μm

Finally, AH neurons were seeded at low densities to minimize the reverse-shooting of AH axons. This strategy yielded a first proof of concept of the reconstruction of the perforant pathway. However the control of the connectivity was not complete, and the yield in operational cultures rather low. This prompted us to try another approach using guiding micro-patterns, presented in the next chapter.

2.4 Further explorations and perspectives

2.4.1 Neuro-degenerative studies in Bernard Brugg team

In the lab in Jussieu, Bernard Brugg and Jean-Michel Peyrin main interest was to study neuro-degenerative processes *in vitro*. PhD students, Sébastien Magnifico, Bérangère Deléglise and Benjamin Lassus, a post-doc Devrim Killinc, a Maitre de Conference Eric Duplus, and engineers, Vanessa Soubeyre and Sandrine Ehret, Morgane Belle, used various microfluidic chips to investigate neuronal degeneration under different toxic stress. I was involved in this work by being in charge of the design and fabrication of the chips needed for these studies (fig. 2.18). I show here some of these studies to illustrate the variety of application of the techniques developed.

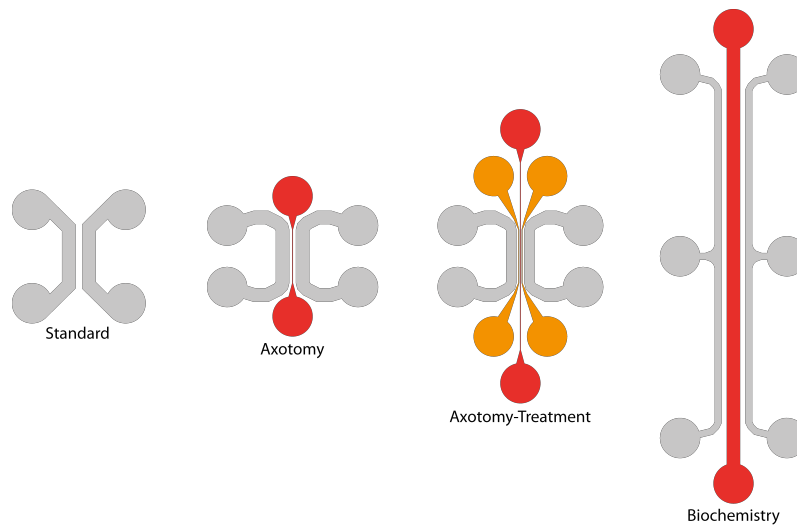


Figure 2.18: Examples of designs of chips used for neurobiology studies.

Sebastien Magnifico investigated $A\beta$ peptide toxicity on compartmentalized populations of neurons. Among others, he explored the different signalization pathway involved in neuronal degeneration after $A\beta$ somatic or axonal treatment. Eric Duplus and Devrim Killinc used chips with a special elongated design to do molecular biology study on compartmentalized neurons.

Bérangère Deléglise extensively used 2C Cx-Striatum networks to study synaptic degeneration and protection following axotomy. For this purpose a special design was used, with an additional narrow macro-channel situated in the middle of the micro-channel array, allowing fast, localized and transient fluid delivery (fig. 2.19). By flowing a detergent (such as Triton) in this additional medium channel, the axons of the input population of the network can be cut (fig. 2.20). By immunostaining or live imaging, Bérangère followed synaptic loss, as well as pre-synaptic and post-synaptic degeneration following this axotomy (accepted paper in appendix A).

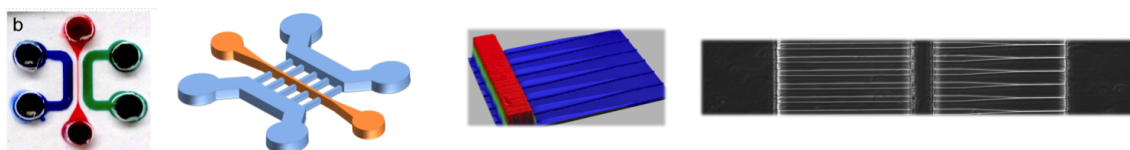


Figure 2.19: Design of the chips used for axotomy studies. *From left to right: Picture of the whole circuit with the three parallel chambers filled with inks ; 3D scheme (not to scale) ; 3D reconstruction by optical profilometer ; translight picture.*

In addition, Bérangère Deléglise further characterized and used 2C Cx-AH network to study the transynaptic toxicity of $A\beta$ peptide. The number of synapses

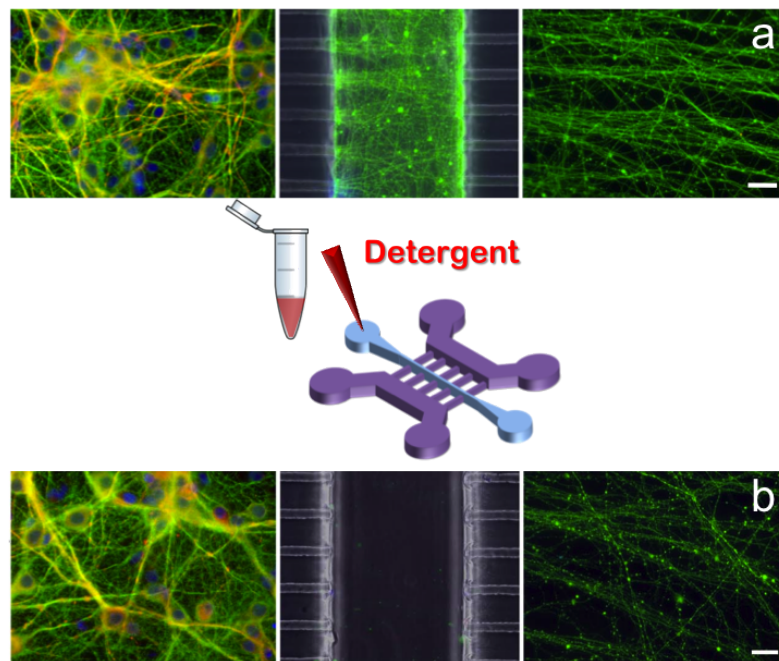


Figure 2.20: Principle of detergent axotomy in the chips. *From top to bottom: (a) DIV6 Cx cultures prior to axotomy immunostained with α -tubulin (in green), MAP-2 (in red) and nuclei (in blue) ; 3D scheme of detergent injection in the central chamber ; (b) DIV6 Cx cultures after axotomy immunostained with α -tubulin (in green), MAP-2 (in red) and nuclei (in blue). Scale bars 20 μ m. Photos credit: Bérangère Deléglise.*

between cortical fibers and AH dendrites was followed after injection of $A\beta$ peptide in the Cx chamber and a synaptic loss was observed (fig. 2.21) (submitted paper in appendix XX).

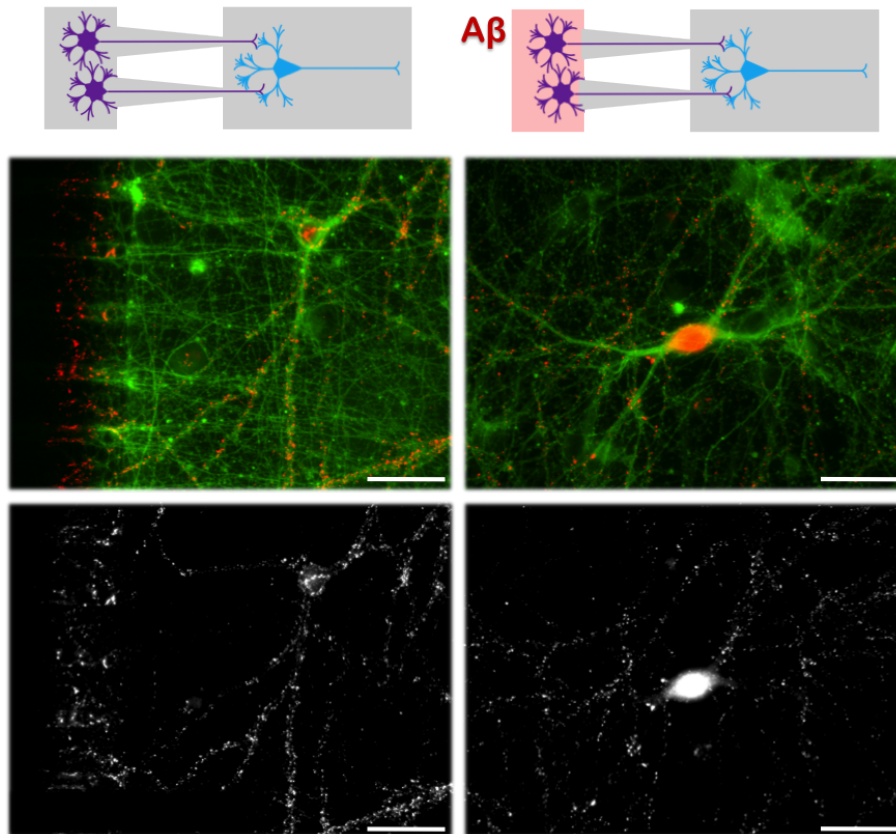


Figure 2.21: Cx-AH synaptic loss following $A\beta$ peptide injection in Cx chamber. *Top part: scheme of the network and the treatment applied ; medium part: 3D scheme of detergent injection in the central chamber ; (b) DIV10 AH cultures innervated by Cx axons immunostained with β -tubulin (in green), synuclein (in red) ; bottom part: immunostaining of synuclein. Scale bars 50 μ m. Photos credit: Bérangère Deléglise.*

2.4.2 Possible ways to explore 3C networks

Studying the propagation of lesions along neuronal networks

Neuro-degenerative studies has already been performed on the 2C oriented networks Cortex-Hippocampus and Cortex-Striatum. Using these 2C networks, the transmission of stress signals or lesions (such as amyloid- β plaques) through oriented synaptic connections can be investigated. However, to study if Alzheimer's lesions propagate in the brain following the connectivity of the neuronal networks, the 3C neuronal networks mimicking the perforant path would be more relevant. Having

three-populations connected in a row, would allow to infect the first population with A β plaques and observe the effect on the two following synaptic connections. In this configuration, the second synaptic connection would probably represent better physiological conditions because none of the connected neuronal populations would have been perturbed externally. By increasing the complexity and biological relevance of the networks, we can get more insight on neuro-degenerative processes as complex as neurological diseases.

A great advantage of using *in vitro* neuronal networks, as compared to *in vivo* or *ex vivo* (slices) approach, is that we can play with parameters that are hardly tunable *in vivo*. In 2C, but even more in 3C neuronal networks, the topology of the synaptic connections can be changed to be different from the physiological one to test new hypothesis. In addition, chimeric networks can be built with some populations of neurons coming from Alzheimer's transgenic mice and some other population coming from wild-type mice. In the perofrant pathway, it would be interesting for example to see if having wild-type DG neurons in the middle of cortex and ammon's horn transgenic neurons would stop or slow down the propagation of Alzheimer's lesions.

Studying the activity of the 3C network

The cortex-hippocampus networks are the core of our memory formation and storage [25, 26]. The mechanisms that underlies these processes has been long studied both *in vivo* and *in silico*. However, if the interplay between the activity of the cortex and hippocampus is well known during active states, this relationship has proven difficult to disentangle during sleep [27–29]. Our *in vitro* neuronal networks that are functional and recordable by calcium imaging, could constitute a powerful model to study this open question. Discussions with Jean-Michel Peyrin and Karim Benchenane started in this direction but time was lacking to pursue this topic.

The method that has been developed together with Renaud Renault to stimulate and record neurons activity simultaneously just by optical methods (refer to chapter 4) could also be interesting for the study of networks rythm. A recent paper has shown that, even when culture randomly *in vitro*, the activity of neurons can either mimic a sleep state, if the culture is not stimulated, or a active state if the activity of the neurons is triggered externally [30]. Thus it seems possible to compare active and sleeping states *in vitro* and thus approach cognitive processes *in vitro*. Given the importance of the topology of neuronal networks *in vivo* the control we can have on it in our microfluidic systems seem promising for future studies.

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Chapter 3

Micro-Patterns to Control Neuronal Outgrowth

3.1 Our approach and challenges

The original aim of the following study was to overcome the limitations of “axon-diodes” and prevent AH neurons to grow in the blocked direction of the diodes. The questions raised by the first preliminary studies lead us to extend the study and explore neurite guidance in a more general context.

At first we tried different geometries of micro-channels. The idea was to find a geometry that would lead different neuronal populations to grow differently. For example we wanted to find a geometry that would block AH neurons in one direction and would let Cx or DG growing in the opposite direction. Such a geometry was difficult to find and we decided to explore more diverse micro-patterns, namely micro-contact printing (uCP), micro-grooves (uGro) and in-mold patterning (iMP), which combine the chemical and mechanical constraints of uCP and uGro.

For each of these three techniques we explored several geometries of patterns from stripes to squares and circles. The study evolved into an exploration of a toolbox of micro-patterns and quantification of their ability to build neuronal networks in microfluidic systems based upon three main criteria.

Our approach was empirical and remains somewhat preliminary. We always kept in mind that we aimed at building neuronal networks and that the techniques used should keep simple to allow large chips production that are needed for molecular biology studies.

3.2 Quantitative Comparison of Micro-Patterns to build Complex Neuronal Networks in Microfluidic Systems

Note: The following section is a publication in preparation for submission.

3.2.1 Abstract

Neuronal networks are the basic processing and architectural bricks of our brains. Neuronal networks have been extensively studied both *in vivo* and *in silico*. Since a few years, functional neuronal networks can also be reconstructed and studied *in vitro*. Despite the advances of micro-patterning techniques to guide neuronal outgrowth, it is still complicated to control the synaptic connections between more than two neuronal populations. Controlling neuron connectivity at the single neuron level is an even stronger and unmet challenge today. In order to progress towards these aims, we explore three micro-patterning techniques combined with different geometries of patterns and quantify their abilities to guide neurons into networks in microfluidic chips. We developed quantitative methods to assess the capacity of micro-patterns to (i) position cell bodies, (ii) orient neurites and (iii) polarize neurons. There was no universal best technique-geometry combination, but we described and evaluated a toolbox of techniques and geometries that can be used to build complex neuronal network *in vitro*.

3.2.2 Introduction

Neuronal networks are the basic computing bricks of our brains. Neurons cannot compute or even survive on their own. Neurons are always assembled *in vivo* to form various neuronal networks and pathways with highly controlled topologies. These topologies have been extensively used as an inspiration to try and build artificial neuronal networks *in silico* but were for a long time difficult to reproduce *in vitro*. Until recently neuronal networks build *in vitro* were either random or patterned with micro-contact printing [1–7]. These networks were limited because they could not control the orientation of synaptic connections.

In 2005, Taylor et al. presented a way to compartmentalize neuronal cultures in microfluidic chips with two culture chambers separated by an array of micro-channels blocking somatic migration but allowing axonal outgrowth [8]. This design opened new routes for the reconstruction of oriented neuronal networks. Different strategies were adopted by different teams. Taylor et al. took advantage of the difference in length between axons and dendrites [9]. We designed asymmetrical

micro-channels called “axon-diodes” [10]. These funnel-shaped micro-channels allow axons to grow only from their wide entrance to their narrow exit. Using this technology, several oriented neuronal networks with diverse neuronal populations (cortex, striatum or hippocampus) were successfully built and used to study degenerative processes [11–13]. Other strategies also emerged to build oriented neuronal networks *in vitro* [14–20]; however, no oriented network with more than two populations has yet been built. With more than two populations, the number of possible topologies of synaptic connexions is considerably increased and controlling them precisely is challenging. In addition, different neuronal subtypes have different physical properties (growth rate, spatial extension, neurite branching) that makes it difficult to find universal means to constraint them. Some neuronal populations also present specific synaptic connexion patterns such as self-connexions. Controlling all these parameters is an ambitious goal but it is necessary to build neuronal networks with fully controlled topologies.

To go beyond the limitations recalled at the beginning of this section, and progress towards the construction of more and more controlled and complex networks, it is important to find tools to control (i) cell positioning, (ii) neurite orientation and directionality, and (iii) neuronal polarization (differentiation between dendrites and axons). To build complex oriented neuronal networks *in vitro*, we need to find techniques that combine these three features. In addition, if we want that these reconstructed neuronal networks can be used for physiological or molecular studies, we need to find simple techniques that allow high-throughput production.

Many studies exist on micro-patterning techniques to control neuronal outgrowth. Micro-grooves and micro-contact printing techniques were used to control cell positioning and/or to constraint neurite orientation [18, 21–29]. However these studies are diverse both in the techniques explored and in the type of neurons or model organisms used. Thus, a more systematic view on how to position cell bodies and how to orient neuronal outgrowth is still lacking. In addition, few studies investigate the issue of neuronal polarization. A recent study have shown that it is possible to use micro-contact printing to polarize neurons [30] but there is still a need to enlarge the number of techniques that allow such polarization.

In this study we explore three micro-patterning techniques: (1) micro-contact printing (uCP), (2) micro-grooves (uGro) and (3) in-mold patterning (iMP). This recently introduced technique consists in moulding a micro-structured object onto a “master” with a protruding microscale or nanoscale pattern, said pattern presenting on its upper surface functional molecules. It can thus be seen as a combination of micro-moulding and micro-contact printing in one step; leading to guiding structures combining biochemical signalling and physical constraints. To our knowledge, it has never been applied to neuron culture so far. These three patterning methods have

been implemented in three main families of geometries: (a) stripes, (b) squares and (c) circles. Diverse size of patterns for each geometry are also considered. The different patterns formed are quantitatively compared regarding their ability to build complex neuronal networks *in vitro*. In other words, we wanted to assess how efficient each of the different geometry-technique pairs were to (i) position cell bodies, (ii) orient neurites and (iii) polarize neurons.

3.2.3 Materials and Methods

Micro-patterning techniques and geometries

Three soft-lithography micro-patterning techniques combined with three type of geometries were explored (fig. 3.1 and fig. 3.2). The nine different combinations explored are called geometry-technique pairs.

uCP protocol was inspired by the one published by Whitesides team in 2010 [31]. The masters were produced by photo-lithography with the negative resist SU8-2005 as previously described [10]. Poly-dimethyl-siloxane (PDMS) was casted on the molds to obtain 5 μm height structures. The structured PDMS was then used as the “stamp”. The stamps were silanized with (tridecafluoro-1,1,2,2 tetrahydrooctyl)trichlorosilane 97% in vapor size. This treatment increased spatial resolution of the patterns. A solution of FITC labeled poly-L-lysine (PLL-FITC) at 100 $\mu\text{g}/\text{mL}$ was incubated for 1h at RT on a flat PDMS surface. This PDMS bulk coated with PLL-FITC served as the “inker”. The stamp was putted in contact with the inker for 2 min and subsequently putted in contact with a cleaned glass coverslip activated by air plasma during 2 min. Stripes, square spots or circle spots of PLL-FITC were obtained depending on the geometry used. The control surfaces for uCP were glass surfaces homogeneously coated with PLL-FITC (10 $\mu\text{g}/\text{mL}$ aqueous solutions incubated overnight at 37°C).

To obtain the micro-grooves PDMS was spin-coated (at 800 rpm during 30 s) and cured on top of the silicon masters. The PDMS thickness obtained was around 200 μm and the grooves were all 5 μm height. After molding the thin and structured PDMS membranes obtained were deposited on top of a glass coverslip to increase their rigidity and facilitate subsequent manipulations. Linear grooves, squared wells or circular wells were obtained depending on the geometry used. The control surfaces for uGro were non-structured thin PDMS membranes obtained by spin-coating the PDMS on a naked silicon surface.

The iMP method was a combination of the uCP and uGro methods inspired by the publication from Biancardo et al. [32]. In this method we used the same silanized stamps and inker as in the uCP method. After the inking step we spin-coated PDMS on top of inked stamps. After curing and unmolding we obtained thin PDMS membranes with grooves coated with PLL-FITC only at their bottom. These membranes were then deposited on top of glass coverslips. Linear grooves, squared wells or circular wells only coated at their bottom were obtained depending

on the geometry used. The control surfaces for iMP were the bottom of very large grooves.

Microchips fabrication

The micro-patterned surfaces obtained were then integrated in microfluidic chips. PDMS blocks of 4-5 mm height with two parallel channels (1 mm width $\times$ 55 μ m height) were aligned on top of the micro-patterned surfaces. To assemble the chips, the surfaces without PLL-FITC micro-patterns were first treated with air plasma for 1 minute. Then the two parts of the microchips were bonded and filled with poly-D-lysine (PDL) at 10 μ g/mL in phosphate-buffer-saline solution (PBS 1X) in the case of micro-grooves, or with PBS 1X without PDL for the other micro-patterns. All microchips were incubated overnight at 37°C. The microchips were rinsed with DMEM before seeding the cells.

Primary cultures

Animal care and experiments were conducted in accordance with standard ethical European Committee Guidelines on the Care and Use of Laboratory Animals. Experiments were approved by the local ethics committee and were performed in an authorized establishment (Trouillet facility licence B75-05-18) under the supervision of authorized investigators. Primary cultures were prepared using mice cortical, striatal or hippocampal neurons extracted and dissociated respectively from E14 or E16 embryonic brains using the protocol described in Peyrin et al. [10]. Timed pregnant OF1 mice at E14 or E16 were purchased from Charles River (Charles River, Lyon, France). All steps of dissection were realized in Gey's balance saline solution (GBSS) supplemented with 0.1% glucose (Life Technologies, Inc., Gaithersburg, MD, USA). Dissected structures were digested with papain (20U/ML in DMEM, Sigma; St. Louis, MO, USA). Papain was then inactivated by fetal bovine serum (FBS, PAA, Piscataway, NJ, USA). Digested tissues were then mechanically dissociated with a pipette in presence of DNase I. After two rounds of rinsing and centrifugating cells were re-suspended in DMEM (Life Technologies, Inc., Gaithersburg, MD, USA). Dissociated neurons were seeded in the microchip at a concentration of $45 \cdot 10^6$ neurons/mL. After few minutes of incubation at RT, allowing neurons to attach to the PDL substrate, complete medium (DMEM 10% FBS with B27 and N2 supplements) was flown in the chambers. The cultures grew for 5 days in a 37°C incubator with 5% CO₂. The cultures were then fixed by para-formaldehyde (4%) during 10 minutes.

Imaging methods

After fixation the cultures were incubated for 30 min with a permeation and saturation solution containing Triton-X100 (0,1%, Sigma; St. Louis, MO, USA) and Bovine Serum Albumin (BSA 3%, Sigma; St. Louis, MO, USA). Primary

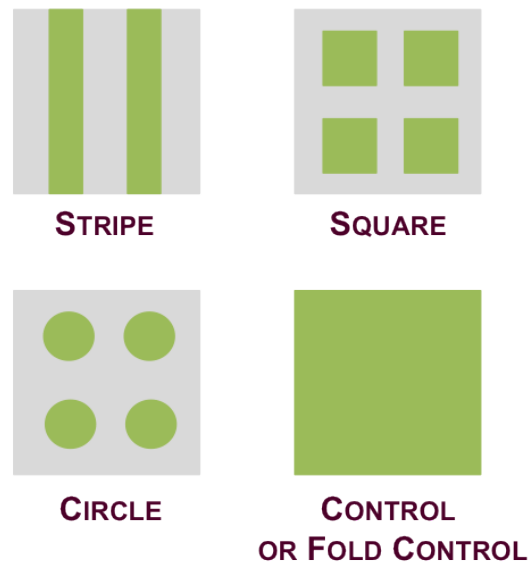


Figure 3.1: Schematic representation of the geometries explored with the example of the micro-contact printing technique.

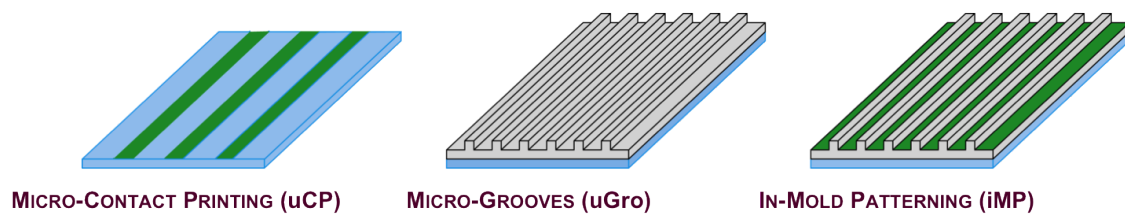


Figure 3.2: Schematic 3D representation of the three micro-patterning illustrated for the stripe geometry.

antibodies directed against β -III tubulin (expressed in the whole cytoskeleton of neurons) and MAP-2 (microtubule-associated-protein 2, mostly expressed in the dendrites of neurons) were incubated for 1h at RT. Finally solutions containing secondary antibodies to stain β -III tubulin in green or red (AlexaFluor 488 or 546) and MAP-2 in blue (AlexaFluor 350) and Hoechst (1/100 000 X) were incubated for 2h at RT or overnight at 4°C. After each incubation the microchips were rinsed with a flow of PBS (1X) during 10 min. For a better conservation the microchips were then filled with SlowFade™ Gold Antifade Reagent. All the antibodies and products came from Life Technologies (Life Technologies, Inc., Gaithersburg, MD, USA). Pictures were taken on a Nikon epifluorescence inverted microscope equipped with LEDs at the Nikon Imaging Center of the Curie's Institute.

Image analysis

Cell positioning analysis

To estimate the density of neurons, fluorescent images of the nuclei (stained by Hoechst) were analysed with a customized Matlab (Mathworks, Inc., USA) code using the Image Processing Toolbox. This program counted only living neurons. The background of the images was first subtracted, then the images were thresholded using Otsu method and made binary. The program then detects all convex particles and calculates the area of each of them. Each particle area was then divided by the mean area of all the particles of the image. The total number of nuclei was obtained by adding the rounded result of this division. Given that the distribution of the particles area is highly centred around the mean, this method allowed to discard all the small particles that are fluorescent particles or dead nuclei. It also takes into account that there are clusters with several neurons on some images. To evaluate the efficiency and specificity of capturing cell bodies in the different geometry-technique pairs, the ratio between the number of nuclei inside vs outside the patterns were determined by manual counting. The efficiency of capture of the cell bodies by a given pattern is defined as the density of cells per unit area on this pattern normalized by the density of cells per unit area on the corresponding control surface. The specificity of capture was defined as the ratio between the density of neurons per unit area inside the pattern and density of neurons per unit area outside the pattern.

Extracting geometry parameters

Because the geometries of the patterns were diverse, in order to keep data analysis time reasonable, and to minimize human bias and errors in the analysis, it was necessary to detect geometrical parameters automatically. The four types of geometries (controls, stripes, squares and circles; fig. 3.1) were classified semi-automatically. The existence of different types of geometries in the analysis, however, also required

the choice of “observable averages” that best reflect the effect of structures and patterns on neurons. Our choices in this respect are discussed in the discussion section. Three geometrical parameters characterizing in more details the patterns were detected: the width of the pattern (meaning the width of the stripe, the length of the side of the squares or the diameter of the circles), the gap between two patterned surfaces (meaning the distance between two adjacent stripes, squares or circles) and the angle of the pattern (meaning the orientation of the patterns on the images with an horizontal left to right oriented line reference for the angles). It is worth mentioning that once these observables have been defined, the analysis is unbiased and valid for all patterns, but the choice of observables itself is not unique, and remains somewhat arbitrary. In particular, different observables may be more or less relevant, depending on the objective of the neuron culture. Translight images (for uGro and iMP) or fluorescent images of the PLL-FITC (for uCP) were analysed, using a specifically written Matlab code, using the Image Processing Toolbox, and based on automatic detection of lines and circles using the Hough Transform. Because of the tube-like shape of neurites, the algorithm had difficulties to differentiate between the edges of the patterns and the neurites, leading to low accuracy for some images. The program was thus designed to be semi-automatic so that the user could check if the detection was accurate or not, and if not he/she could measure the width, gap and angle of the patterns semi-automatically (by drawing lines on the picture). 50% of the images were treated fully automatically reducing substantially the analysing time for detecting the geometry parameters.

Orientation analysis

To quantify the orientation of the neurites, fluorescent images of the cytoskeleton (β -III tubulin or MAP-2) were analysed using a customized Matlab code, using the Image Processing Toolbox, and inspired by methods previously published and based on the two dimensional Fast-Fourier Transform (2D FFT) [33–35]. This method allowed us to calculate the percentage of the intensity of the images oriented along a given angle $\pm 1^\circ$. The images were cropped to the largest square. The threshold of the images were calculated by Otsu method and edges were detected using Canny filters to erase cell bodies. A 2D FFT algorithm was then applied. The power spectrum was calculated and normalized by the size of the image. The lowest 1% and highest 30% frequencies were cut because they were not relevant for the analysis. The lowest frequencies correspond to patterns that are located at the edges of the pictures. The highest frequencies correspond to single neurite events that are noisy and correspond to small persistence length. The total intensity of the image oriented around a given angle $\pm 1^\circ$ was calculated by adding all the intensities of the power spectrum masked with a 2D Von Mises function centred around this given angle. We got histograms showing the percentage of the intensity of the images oriented along all different angles, normalized by the size and total intensity of the image.

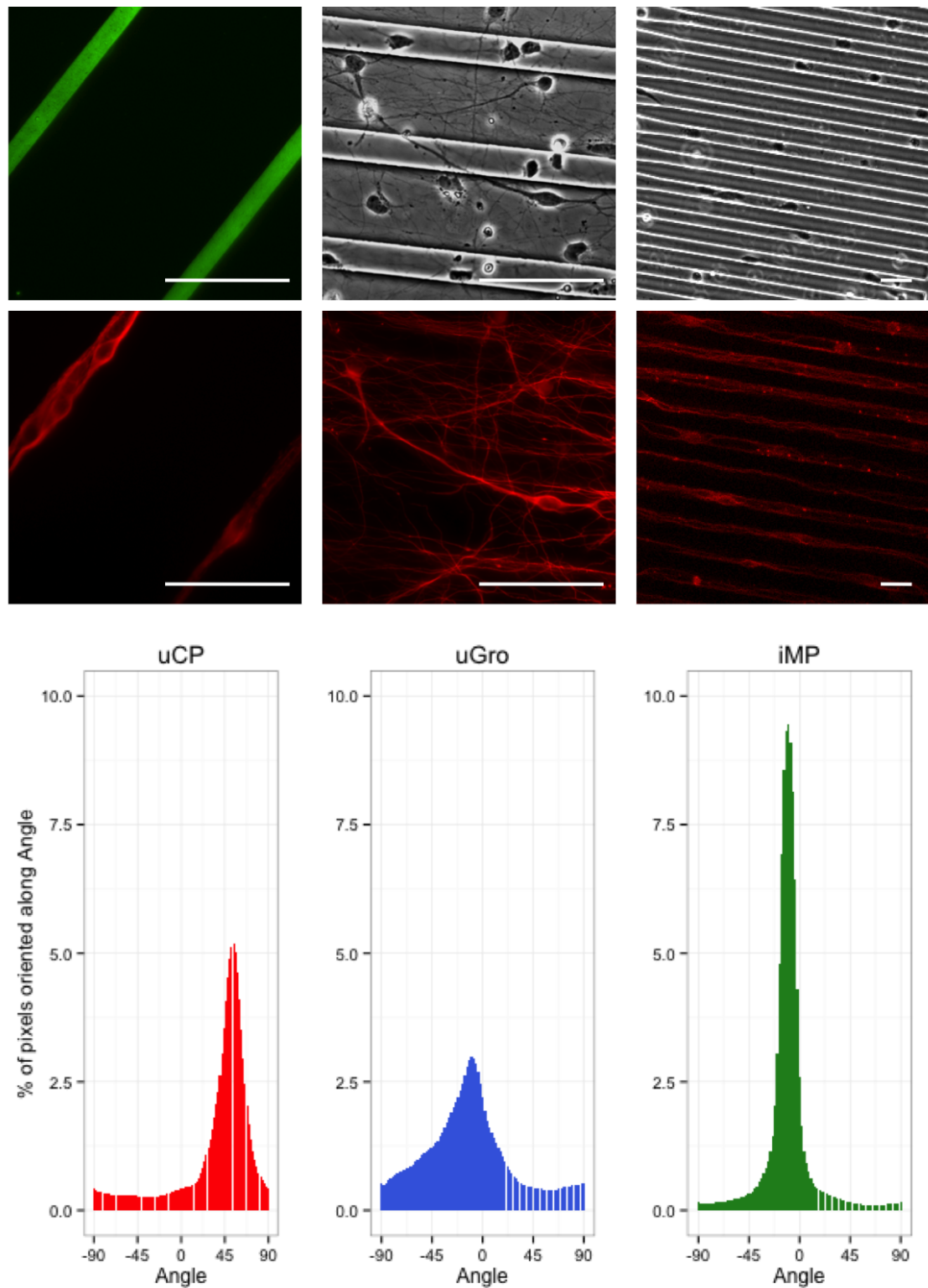


Figure 3.3: Example of images and histograms for the three techniques (uCP, uGro and iMP from left to right) with the example of the stripe geometry. *Top line: green fluorescent image showing PLL-FITC and translight images. Medium line: red fluorescent images showing β -III tubulin. Scale bars 50 μ m. Bottom line: histograms obtained by the FFT analysis.*

Depending on the type of staining, these histograms represented the percentage of neurites or dendrites (β -III tubulin or MAP-2) that are oriented along all possible angles (fig. 3.3). The reference for the orientation angles was the angle of the pattern (equals to 0°). The angles range from -89° to $+90^\circ$ (the information in the FFT is by definition symmetric by a rotation of 180°). See figure 3.18 for an overview of the steps of the customized Matlab code and figure 3.19 for an illustration of the main analysis steps on two image examples.

From the histograms, we extracted for statistical analysis the angle, width at mid-height and area of the highest peak. The area of the highest peak correspond to the sum of all values of the histogram with an angle comprised around the angle of the maxima $\pm 14^\circ$. This area is called the index of orientation of the culture. The width of the peak at mid-height is referred as the dispersion of the orientation. See figure 3.21 for illustration.

For the square and circle geometries the orientation along a given angle x and the orientation along the angle $x + 90^\circ$ were equivalent due to the symmetry of these geometries. In consequence histograms were folded by a rotation of 90° and the angles of orientation analysed were only ranging from -44° to $+45^\circ$. For the control geometry the analysis were done with both folded and unfolded histograms.

Statistical analysis

All statistical analysis were conducted using program R [36]. Linear models (lm function in R) were used to assess whether the differences observed were significant or not. The means and standard error of the means were plotted. Akaike's Information Criterion (AIC) was used to determine the support of each model to explain the data.

3.2.4 Results and Discussion

Positioning cell bodies

The different geometry-technique pairs were first tested for their capacity to capture the cell bodies of neurons. The efficiency of capture (density of cell bodies per patterned unit area normalized by the control) and the specificity of capture (ratio between the density of cell bodies inside and outside the pattern) were evaluated.

As expected the uCP and iMP techniques were by far the most specific, reflecting the fact that the adhesive PDL coating was homogeneous for the uGro technique (fig. 3.4). The specificity for all geometries for both uCP and iMP technique is always equal to 1 which means that all captured neurons are inside the patterned surfaces. For both uCP and iMP, the square and circle spots or wells were the most efficient to capture the cell bodies. The stripe geometry seems to be less efficient but it depends width and gap dimensions.

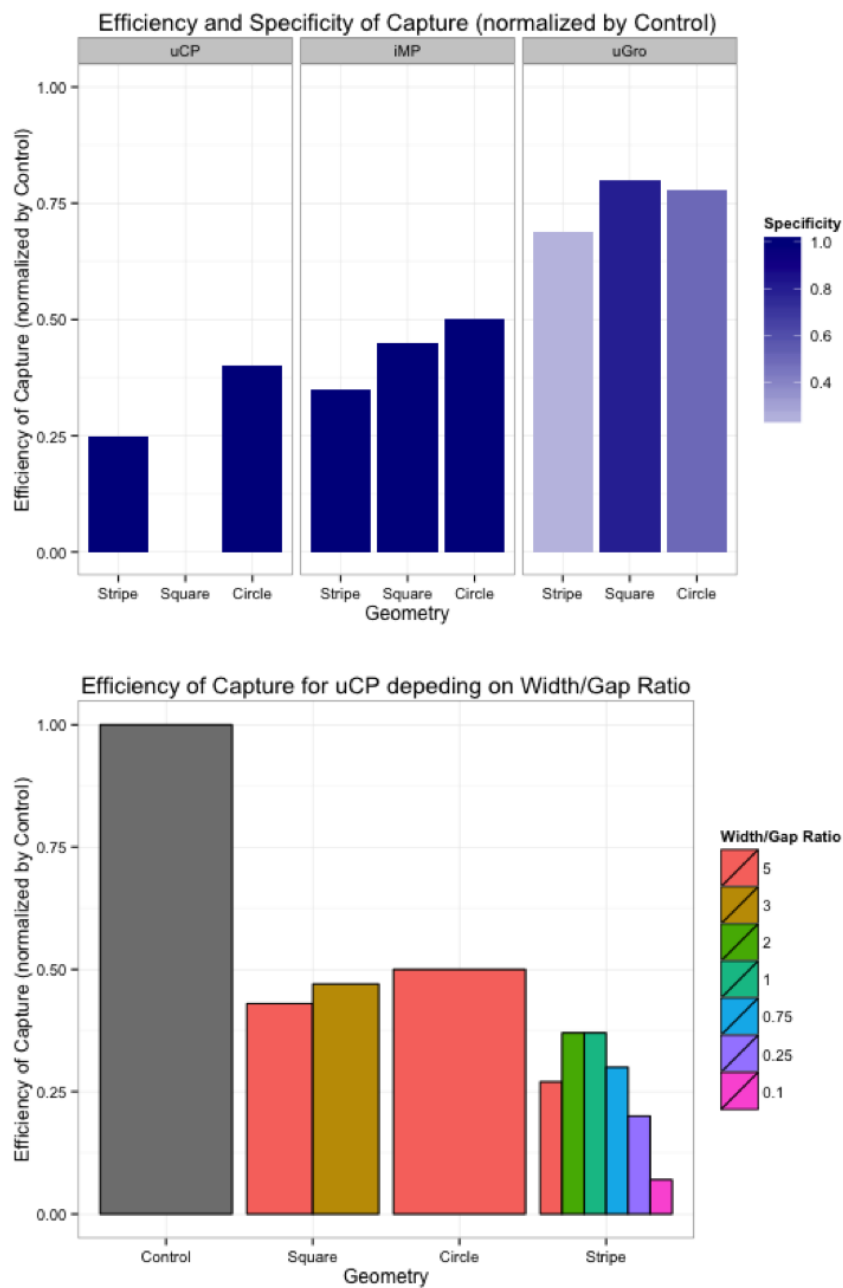


Figure 3.4: Capacity of the micro-patterns to position cell bodies.

Different width/gap ratio were tested for the uCP technique. The efficiency of capture is similar for stripes and squares and circles when their characteristic sizes are similar (width of the stripes $\approx$ side of the squares $\approx$ diameter of the circles). For the stripes we can see that the density of cells captured is decreasing while the width/gap ratio of the patterned surface is decreasing (fig. 3.4). It is more difficult for cells to attach on smaller adhesive surfaces, especially if one of the dimensions of the pattern is smaller than an average cell body diameter ($\approx 10 - 20 \mu\text{m}$). When the gap between adhesive surfaces is increasing, the probability that the cells attach on the pattern is also reduced.

The best combination to capture cell bodies is to use uCP or iMP combined with large patterns and preferentially squares or circles. The size of the patterns can be tuned to capture more or less cells depending on the needs.

Neurites Orientation

The second important parameter in order to control the topology of neuronal networks *in vitro* is the capacity of micro-patterns to orient neurite outgrowth.

Geometry matters more than Technique

An analysis across the whole dataset showed that the best models to explain the variations of the index of orientation as well as the orientation angle was the geometry parameter. Linear models were computed in R evaluating the variation of index of orientation (or orientation angle) depending on geometry only, technique only, an additive term of geometry and technique or an interactive term of geometry and technique. The AIC was calculated for each of these models. For the index of orientation as well as for the orientation angle, the geometry parameter explained more the variations observed in the dataset ($\Delta\text{AIC} > 100$).

Stripe is the best geometry to orient neurite outgrowth

Regarding the index of orientation of neurites, the difference between geometries are larger than the difference between techniques. We can see (fig. 3.5) that the stripe geometry is better to orient neurites than the square geometry, followed by the circle geometry.

Regardless of the technique employed, stripes are the best geometry to orient neurites outgrowth with an average index of orientation around 40% along the angle of the pattern (fig. 3.5). The index of orientation (percentage of neurites oriented around the angle of main orientation $\pm 14^\circ$) of stripes is twice as big as the one of the control condition and their angle of orientation is significantly lower than in the

control condition.

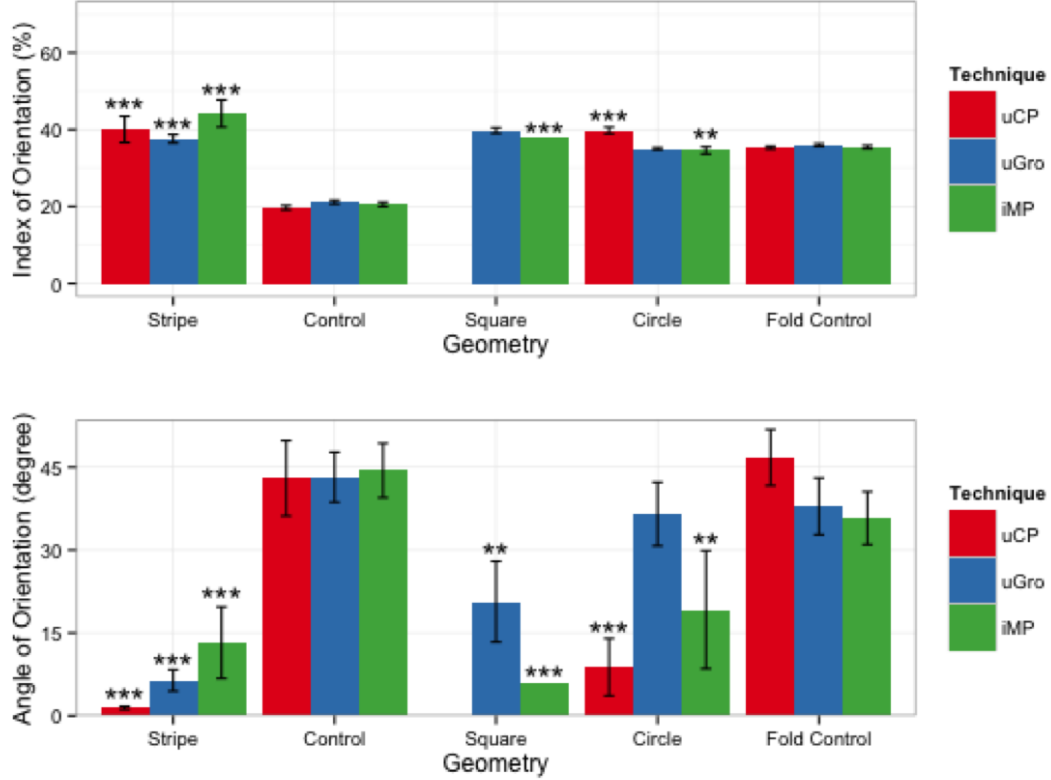


Figure 3.5: Index and angle of orientation of all neurites for all geometry-technique pairs grouped by geometry. The significance of the differences were determine by linear models in R and are relative to the control (for stripes) or to the fold control (for square and circle). *Higher index of orientation depict higher percentage of neurites oriented. An angle of orientation of 0° traduces a perfect orientation along the pattern* (***) for p -value < 0.001).

Neurites follow dihedrals

The index of orientation of the neurons for the stripe geometry is not significantly different for the three techniques explored (fig. 3.5). This result is surprising because in the case of uGro all surfaces allow neuronal adhesion to the substrate. This more permissive technique was expected to be less efficient than uCP and iMP. Previous studies, offering to neurons the choice to grow either on flat surfaces coated with specific adhesive molecules or on structured surfaces coated with non-specific adhesive molecules, showed that neurons preferred mechanical confinement and grew preferentially on the structured surfaces [25]. This may explain why the mechanical guidance by uGro is almost as good as chemical guidance by uCP and iMP.

Nano-grooves were already used to enhance neuronal growth and/or orient neuronal outgrowth [23, 34, 37–42]. With grooves around 100 – 500 nm, two preferential orientations were observed, one along the grooves and the second perpendicular to the grooves [37, 41]. Here, we show that with deeper grooves (5 μm) the number of neurites growing across the grooves is reduced and neurites mainly grow along the grooves.

For uGro and iMP technique, the index of orientation for the square geometry is significantly higher than the control. In addition, for uGro and iMP the angle of orientation is significantly closer to the angle of the pattern than in the control. These results show that neurites are mainly oriented along the pattern angle and that the square geometry is closer to the stripe geometry than to the control case. This suggests that the neurites are not meandering inside the squared wells but are mainly located at the edges of the pattern. Neurites seem to follow dihedrals (fig. 3.6). This result, in accordance with the predominance of mechanical guidance previously shown [25], might also explain the good index of orientation for the uGro technique and the stripe geometry that was previously highlighted.

Neurites cross non-adhesive areas along the shortest path between adhesive areas

For uGro and iMP techniques, we just commented the fact that the square geometry is closer to the stripe geometry. For uCP the circle spots orient the neurites along the angle of the pattern. Neurites are able to cross over two adjacent spots and grow to form squared grids with the points of the grids being centred at the center of the circular spots (fig. 3.7). The fact that the neurites align in two main directions, that correspond to the directions along which the distances between adjacent circles are the shortest, suggests that neurites cross preferentially over the shortest non-adhesive path between adhesive areas. This result has recently been highlighted by Hart et al. [43]

The crossing of neurites over short non-adhesive surfaces might also be explained by the fact that uCP is less efficient to guide neurites than iMP. In the case of iMP the non-adhesive gaps between patterned surfaces are combined with a mechanical constraint. In this case the gaps between patterns cannot be crossed and neurites are completely confined inside the patterned surfaces (see the difference between fig. 3.7 and fig. 3.6 middle column).

Influence of the size parameters of the patterns

In the previous section we already highlighted the fact that neurons can cross over non-adhesive distances but prefer to cross over the shortest non-adhesive distances. There is then a strong influence of the size parameters of the patterns that

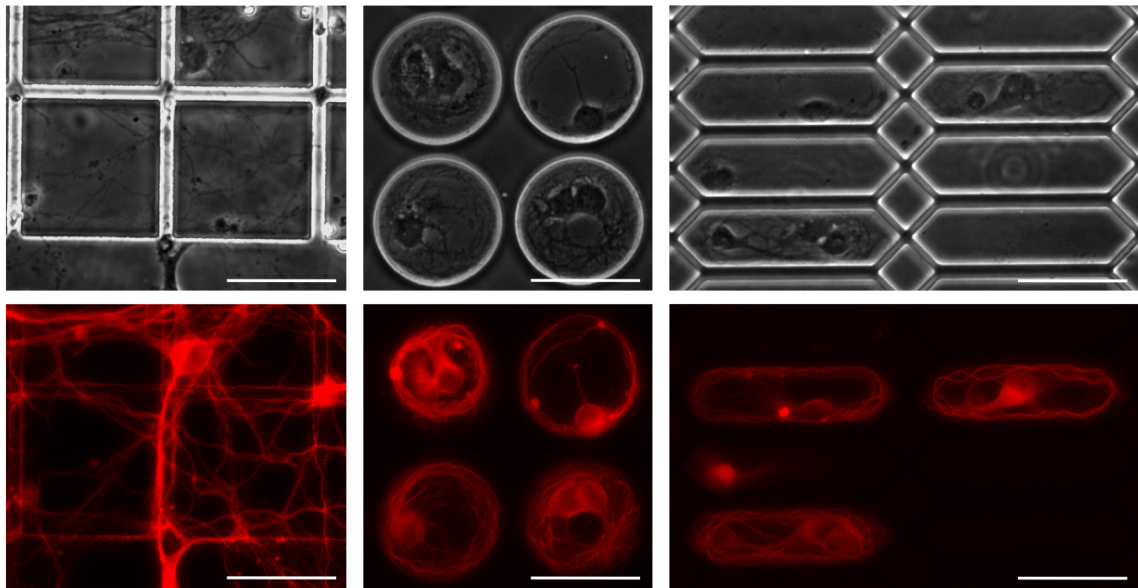


Figure 3.6: Illustration of the fact that neurites follow dihedrals for uGro and iMP techniques. *Top layer: translight pictures. Bottom layer: red channel showing the immunostaining of the β -III tubulin. Left column: uGro-square combination. Medium column: iMP-circle combination. Right column: iMP-hexagon combination. Scale bars: 50 μ m.*

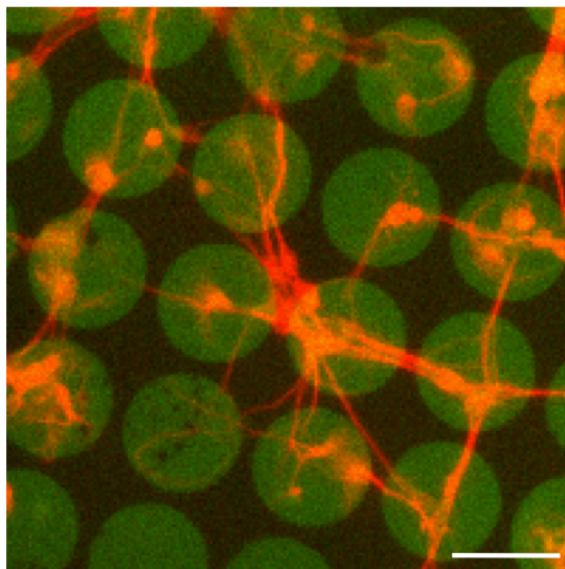


Figure 3.7: Striatal neurons (DIV5) grown on a surface patterned by micro-contact printing with circles of 45 μ m diameter. *Merge of red and green fluorescent channels showing respectively β -III tubulin and PLL-FITC. Scale bar: 45 μ m.*

needs to be explored. Two size parameters were varied, the width of the patterns (width of stripes, side of squares and diameters of circles) and the gap between two adjacent patterns (if this gap is a variable like in circles, the shortest gap was taken as a reference). Very diverse sizes of patterns were explored. To simplify we decided to combine our two size parameters into a single width over gap ratio. To illustrate the influence of this ratio on the orientation of the neurites we present the comparison between the three techniques for the stripe geometry (fig. 3.8).

The index of orientation decreases when the Width/Gap ratio increases showing that smaller width and/or larger gaps are in favour of a better orientation of the neurites. The influence of the gap can be explained by the previous result that neurites can cross small non-adhesive gaps. The influence of the width is due to the fact that larger patterns allow more meandering of the neurites. This is illustrated by the graph figure 3.8 showing the dispersion of orientation according to the Width/-Gap ratio and techniques. This dispersion (i.e. the width of the highest peak of the histogram at mid-height) is increasing when the Width/Gap ratio is increasing. When the width of the pattern increases, neurites have more freedom to orient along angles diverging more from the angle of the pattern.

It is interesting to notice that uGro is less sensitive to the width and gap dimensions than uCP and iMP. This was expected because this technique is more permissive. The depth of the micro-grooves would be more relevant to explain the orientation of the neurites.

Polarization of neuronal outgrowth

The third important parameter to control in building neuronal networks *in vitro* is the polarization of the neurons, meaning that it is important to find a way to orient and direct selectively axons versus dendrites. *In vivo* the topology of neuronal networks is diverse and depends highly upon neuronal subtypes. Building complex neuronal networks implies controlling both inter- and intra-population synaptic connections. In some cases, for example ammon's horn neurons from the hippocampus, it is even important to control the formation of self-synapses. Synaptic connections mostly form between axons and dendrites or between axons and cell bodies. Thus controlling the orientation of axonal and dendritic growth is important to guide them and allow them to meet only at the places of desired synaptic connections.

The immunostaining realized in this study, β -III tubulin and MAP-2, allowed us to observe respectively all neurites or dendrites. The difference between the index of orientation and the angle of orientation between all neurites and dendrites is rather small. However, we can notice that for the stripe geometry, and regardless of the technique used, the index of orientation is significantly smaller and the angle

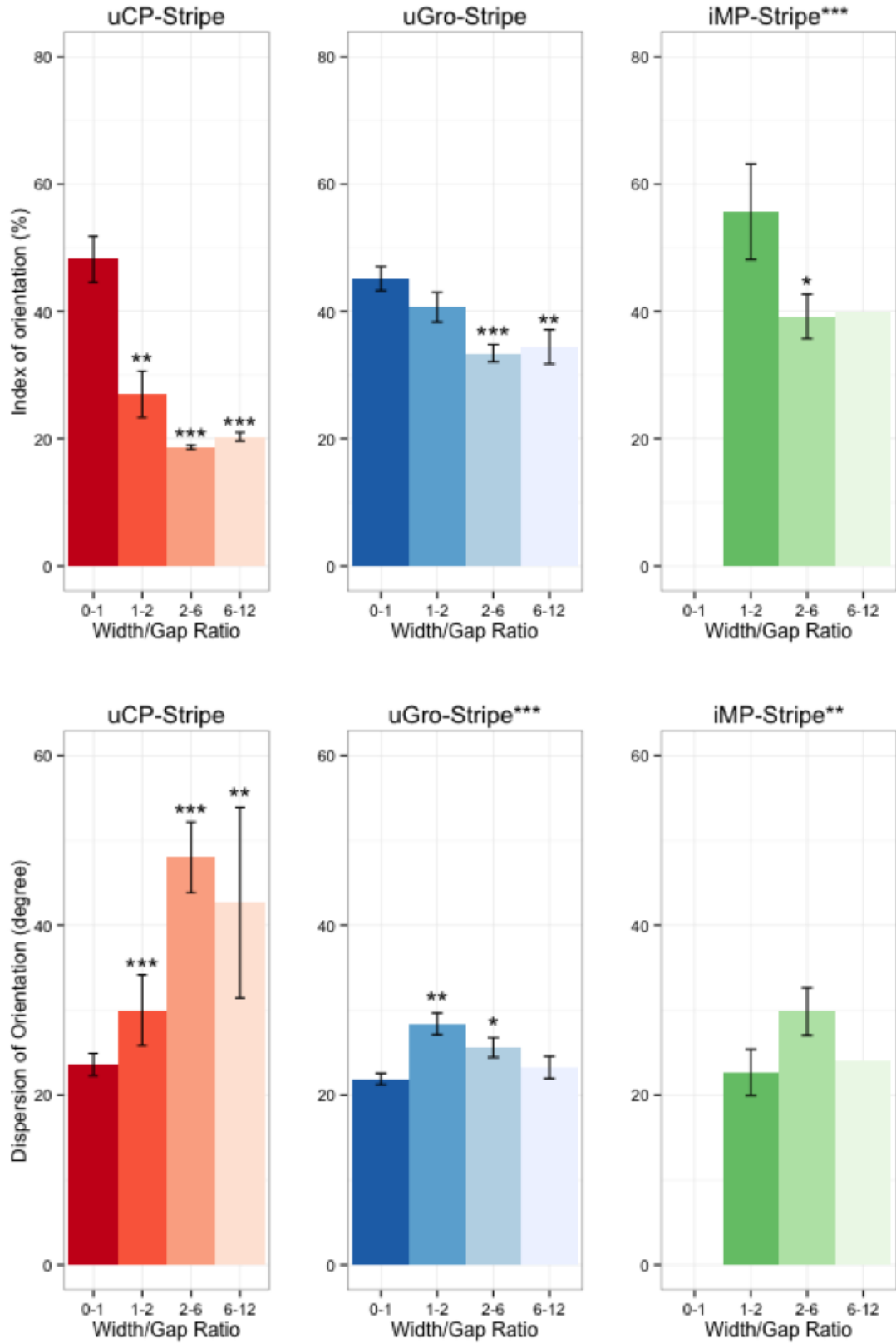


Figure 3.8: Index and dispersion of orientation of all neurites and the stripe geometry depending on technique and Width-Gap ratio. The significance of the differences were determine by linear models in R and are relative to first Width/Gap ratio category (*** for p-value < 0.001).

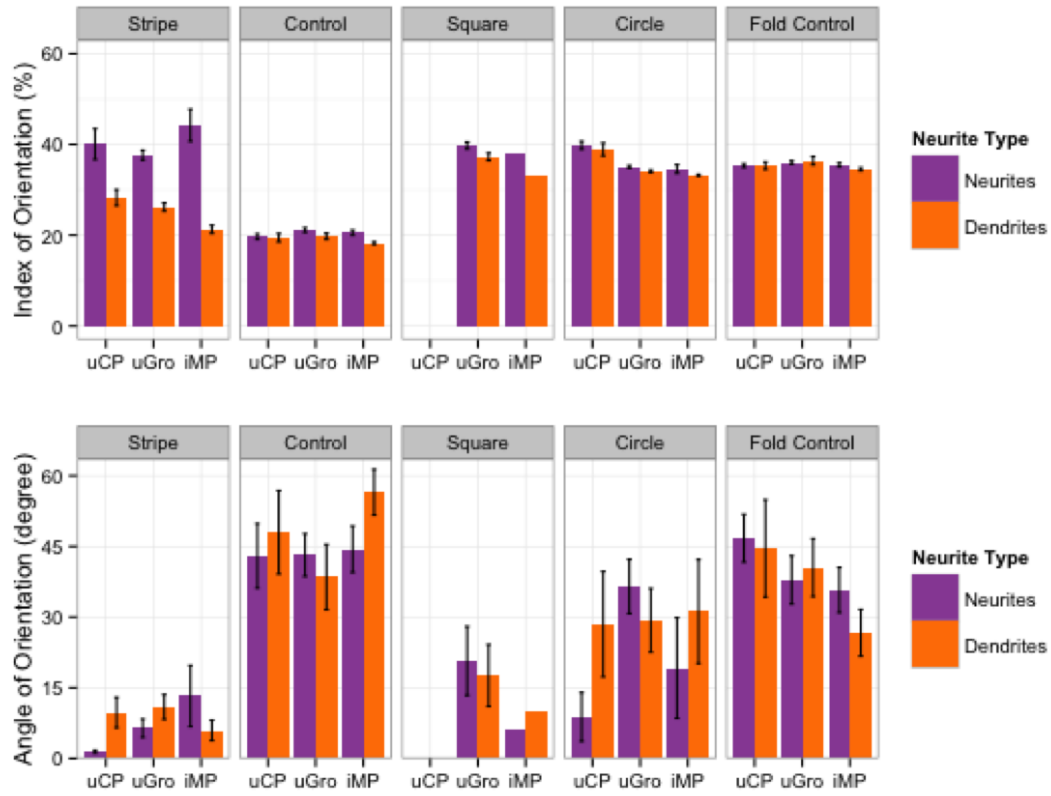


Figure 3.9: Compared index and angle of orientation for all neurites vs dendrites (evaluated by the area and angle of the highest peak of the FFT histograms) for all geometry-technique pairs grouped by geometry (***) for p-value < 0.001).

of orientation is significantly increased for the dendrites as compared to all neurites (fig. 3.10).

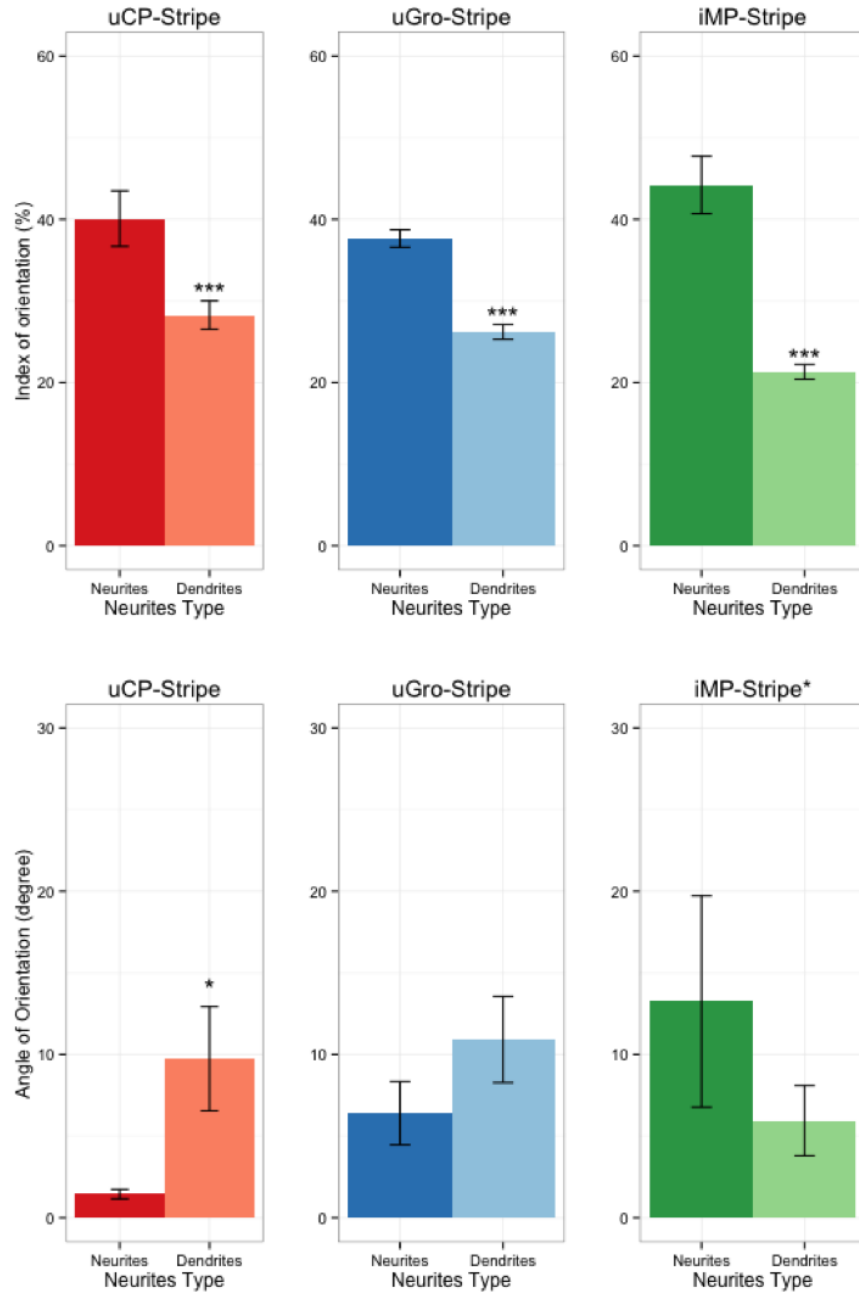


Figure 3.10: Zoom on the index and angle of orientation for all neurites vs dendrites for the stripe geometry.

The differences observed for uCP suggest that the dendrites can cross more easily larger non-adhesive surfaces than axons. The differences observed for uGro suggest that dendrites can more easily grow across the micro-grooves, suggesting that they can climb structures more easily than axons. These results are in accordance with the studies done in C. Villard's lab showing that dendrites can grow more easily on curved adhesive micro-patterns, suggesting that axons are more tensed than dendrites [30].

The only other significant difference observed between all neurites and dendrites is in the case of the uCP-circle combination. In this case the index of orientation is not varying much between all neurites and dendrites but the angle of orientation is significantly increased for the dendrites. This observation reinforces the hypothesis that dendrites can cross more easily larger non-adhesive distances.

Overall dendrites seem to be more “flexible” in the sense that they overpass more easily chemical or mechanical constraints than axons. These results suggest that geometry, technique and dimension parameters could be tuned to exacerbate the axons vs dendrites differences and to control precisely the relative growth directions of axons and dendrites.

Best geometry-technique pairs to build complex neuronal networks *in vitro*

The table of figure 3.11 summarize our findings and recapitulate the efficiency of all the geometry-technique pairs explored regarding (i) positioning of cell bodies, (ii) orientation of neurites and (iii) polarization of neurons. None of these pairs is highly efficient for all three parameters. Each pair as a unique set of capacities.

Efficiency code: poor ok efficient

	Stripe			Square			Circle		
	uCP	uGro	iMP	uCP	uGro	iMP	uCP	uGro	iMP
Cell Positionning				NA					
Neurite Orientation				NA					
Neuronal Polarization				NA					

Figure 3.11: Summary of the efficiency of the geometry-technique pairs regarding the three parameters explored (cell positioning, neurite orientation and neuronal polarization).

uCP and uGro combined with the stripe geometry are probably best to build neuronal networks because they combine cell positioning, good neurite orientation and allow to differentiate the angle of orientation between axons and dendrites. This

last parameter could probably be improved by playing with the size parameters of the micro-patterns (width, gap and even depth for uGro). In addition, in the case of uGro and iMP, neurites are more or less confined in the grooves and that might enhance their resistance to the shear stress that is easily generated in microfluidic systems.

3.2.5 Conclusion

In the field of *in vitro* neurobiology, being able to build complex neuronal networks is of tremendous interest as they constitute the basic processing bricks of the brains. Oriented neuronal networks presenting two neuronal populations have already been reconstructed *in vitro* and were proven functional. However, growing networks with more complex topology or more neuronal populations remain a challenge and very little comparative and comprehensive studies have been made on the micro-patterning techniques available to achieve this goal. In this study we explored several techniques and patterns and developed a method to quantitatively assess their ability to (i) position cell bodies, (ii) orient neurites and (iii) differentiate between axons and dendrites. We show that, depending on which topology of network is targeted different parameters (technique, geometry and size of pattern) can be tuned to achieve the best control possible. More techniques and geometries need to be explored and evaluated with the same image analysis methods to offer a complete toolbox to build complex neuronal networks *in vitro*. One crucial point to investigate next is the differences between neuronal subtypes. The results presented here already offer robust tools to control the architecture of neuronal networks. They are currently being implemented to build a three-population network that mimics the whole mouse hippocampus.

3.3 Detailed explanation of the Orientation Analysis Method

3.3.1 Origins of the Orientation Analysis Method

In neurobiology, the orientation of neurites is often studied in the context of brain development, when growth factors, adhesive extra-cellular matrix (ECM) proteins or even electric fields, are studied regarding their attractive or repulsive effect for neurites outgrowth [44–49]. An increasing number of studies is also devoted to the effect of micro-patterns on neurites outgrowth. In these studies, orientation quantification is generally based on the segmentation of images to reconstruct the neuronal layout from the images. This process is called neurite tracing and is usually semi-automated and time consuming. After this neurite tracing, orientation is performed. The most common way to quantify the orientation of one neurite is to

calculate the angle of the segment joining the origin of the neurite on the cell body to the tip of the same neurite [37, 38, 46, 50]. The angle between the direction of the neurite at its origin and at its tip has also been calculated. Considering the tortuosity neurite can achieve, these methods are not fully satisfactory. In addition, in these reconstruction methods, the analysis is achieved on single neurons: thus they are applicable only to cultures at low density, in which neurites are not overlapping too much. I thus developed an alternative method able to evaluate the global orientation of neurites in a dense culture of neurons.

2D Fast Fourier Transform (FFT) was first used as a directional detector in material sciences to detect the orientation of fibres in textiles, papers or concrete [33, 51–53]. This method was then applied to biology in order to measure the orientation of cells, tissues, blood vessels or collagen fibres [35, 54–58]. Some applications to neuroscience were attempted [59–61], and two plugins called Directionality and OrientationJ were developed for ImageJ/Fiji in 2010 based on this method [35, 59]. However they have not yet been extensively adopted by the community. In addition, pre-processing of the images (contrast adjustment only) and the post-processing of the histograms obtained (Gaussian fit) in these plugins were not appropriate for our datasets. I thus decided to develop an original Matlab code to process the images. The main differences with the existing plugins are the following:

- The use of an edge detection in addition to the contrast adjustment in the pre-processing in order to eliminate signals from cell bodies.
- the use of Von Mises functions to detect intensities as a function of the angle of orientation and smoothen the histogram.
- Elimination of low and high frequencies signals of the FFT.
- Replacement of Gaussian histogram fitting by direct information extraction from the histogram highest peak.

3.3.2 Principle of the 2D FFT of an image

The Fourier Transform is transforming a signal described in space (or time) to its component in the frequency domain. The principle of the Fourier Transform is to decompose a signal into a summation of cosine and sine functions with different frequencies and amplitudes [62–64]. Addition of all the components of the Fourier Transform can yield back the original signal, a process called reverse Fourier Transform. With an infinite summation of sine and cosine it is in principle possible to decompose and/or reconstruct all signals (Fourier Theorem).

The Fourier transform $G(u)$ of a function $g(x)$ can be written as:

$$G(u) = \int_{-\infty}^{+\infty} g(x) e^{-i2\pi ux} dx$$

and Euler's formula gives:

$$e^{-i2\pi ux} = \cos 2\pi ux - i \cdot \sin 2\pi ux.$$

To speed up processing, the continuous integral can be replaced by a discrete summation. We can then write the formula of the Discrete Fourier Transform (DFT):

$$G(u) = \sum_{x=0}^{N-1} g(x) \cdot e^{\frac{-i2\pi ux}{N}}$$

where N depends on the number of sampled points along the function $g(x)$, which are supposed to be uniformly spaced.

Some examples can illustrate the potential of the Fourier Transform (fig. 3.12). One of the simplest example is a sine function. We consider that we have N sampling points and an arbitrary amplitude. The DFT of this signal is simply a peak for a frequency $f = 1$. If we would look at negative frequencies, we would see that there is also a peak for $f = -1$ with the opposite height as the one for $f = 1$ because

$$e^{i2\pi x} - e^{-i2\pi x} = 2i \cdot \sin 2\pi x.$$

The height of the peak in the DFT is linked to the amplitude of the sine. The DFT of a sine with a half period, so a double frequency, and the same amplitude, will provide two peaks at $f = 2$ and $f = -2$ with the same height as before. A flat function $g(x) = C$ with constant C has as DFT a single peak for $f = 0$.

In figure 3.13 a more complex example is shown. The original signal (red curve) is a summation of sine and cosine functions with diverse frequencies and amplitudes (wave functions in blue on the image). In practice, this can be seen as an approximation of a periodic square wave, approaching the perfect square wave for a number of components tending to infinity. The resulting DFT is shown in blue on the right panel.

Now that we have some intuition of what the 1D Fourier Transform and the 1D DFT (meaning the discrete Fourier transform for a mono-variable signal) does, we can approach the 2D DFT that was used here to analyse the orientation of neurites. The 2D DFT decomposes a 2D image into its wave components. These wave components have a given frequency and amplitude (as in the 1D DFT) but they are described by one additional parameter, the orientation of the wave.

The mathematical formula for the 2D DFT is:

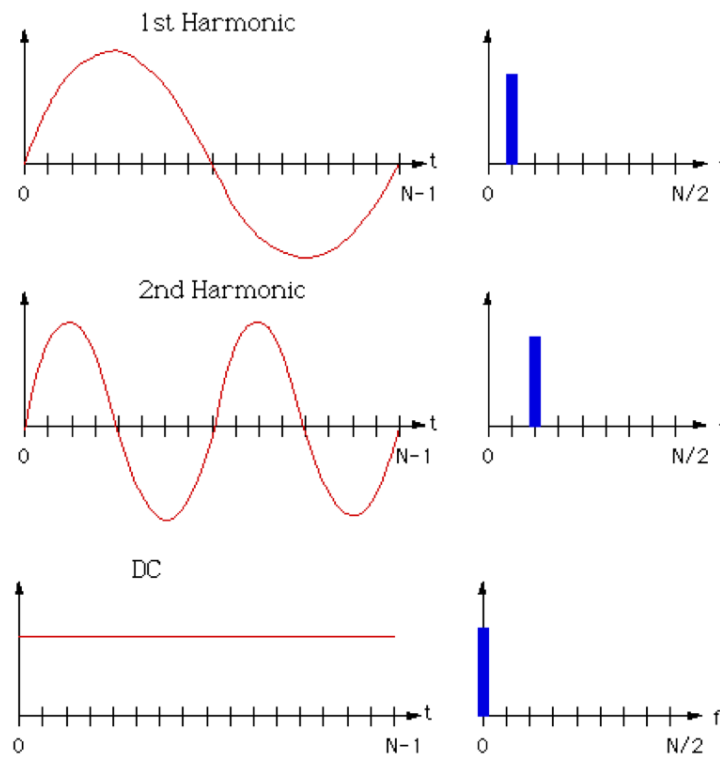


Figure 3.12: Simple mono-variable functions and their corresponding Discrete Fourier Transform. *Image adapted from paulbourke.net.*

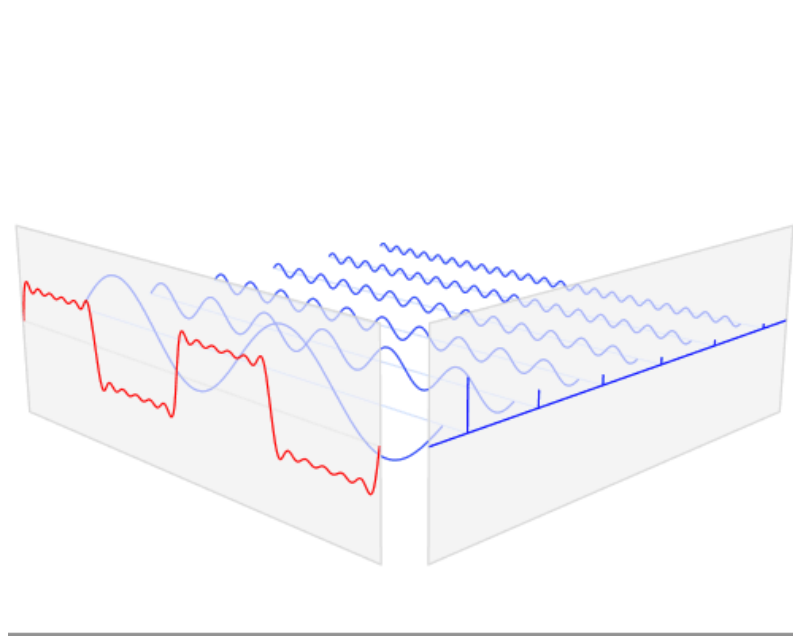


Figure 3.13: Approximate square wave signal with its decomposition in cosine and sine functions and its resulting DFT. Image extracted from a video created by Lukas V. Barbosa for the english Wikipedia page on the Fourier Transform (http://en.wikipedia.org/wiki/Fourier_transform).

$$G(u, v) = \sum_{x=0}^{N-1} \sum_{y=0}^{N-1} g(x, y) \cdot e^{\frac{-i2\pi(ux+vy)}{N}}$$

The function g and its transform G now have two variables (two space variables for g and two frequency variables for G). For an image of size $n \times n$, N is commonly equal to $n/2$. This is due to the fact that you need at least two pixels to build the period of a wave signal. There is also an upper limit to the frequencies encoded in an image of size $n \times n$, called the Nyquist frequency [64].

To understand how this 2D DFT is computed, imagine that we apply a 1D DFT on each row (or horizontal lines) and then on each column (or vertical line) of a 2D image. This algorithm grasping the meaning of the 2D transform, would be very time-consuming for large dataset image processing. The algorithm commonly used in image processing is called the Fast Fourier Transform algorithm. Figure 3.14 displays several sine waves and their FFT. Figure 3.15 shows more complex patterns corresponding to the summation of several sine functions with different frequencies and their FFT.

The result of the FFT of an image is a matrix of the same size as the image filled with complex numbers that are the result of the FFT at each point of the image.

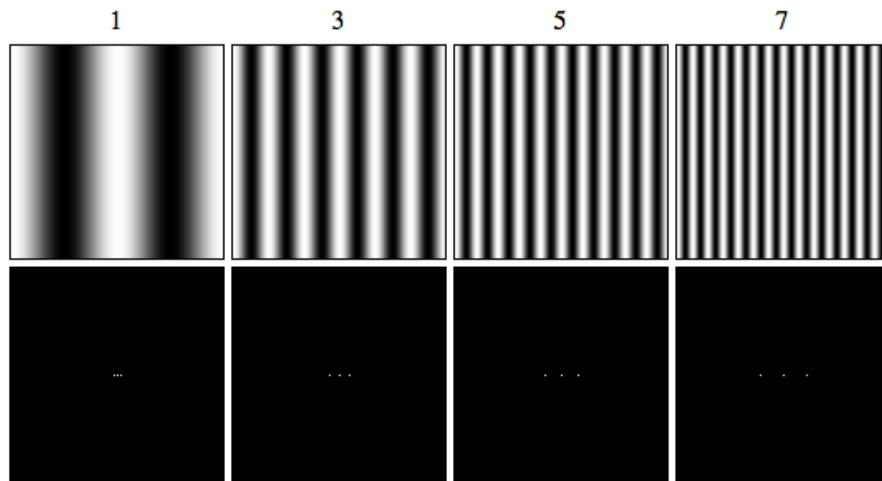


Figure 3.14: Sinusoidal waves with different frequencies and their FFT (power spectrum displayed). Images extracted from a website created by Steven Lehar called “An Intuitive Explanation of Fourier Theory” (<http://cns-alumni.bu.edu/~slehar/fourier/fourier.html>).

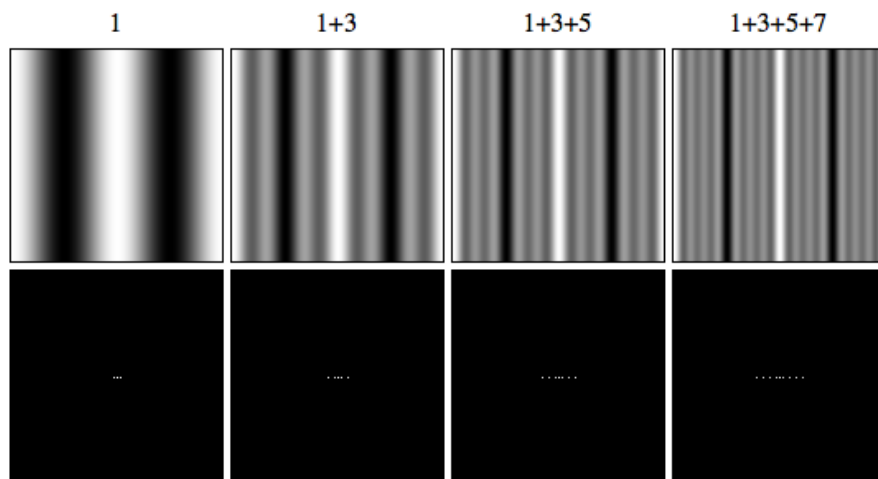


Figure 3.15: Images resulting from the summation of several sinusoidal wave images with different frequencies and their respective FFT (power spectrum displayed). Images extracted from a website created by Steven Lehar called “An Intuitive Explanation of Fourier Theory” (<http://cns-alumni.bu.edu/~slehar/fourier/fourier.html>).

Due to the algorithm, the FFT gives results for which low frequencies are peripheral and high frequencies are central. This is not easy to interpret so the FFT is always “swapped” or “shifted” so that the lower frequencies are then situated close to the center and high frequencies are at the periphery (details below). Complex numbers are not easy to visualize hence the square of the magnitude of the FFT, called the power spectrum (PS), is usually shown. All the FFT shown in this manuscript are the power spectra of their respective swapped FFT.

As can be noticed on the example figures 3.14 and 3.15, the FFT and thus the resulting PS admit a central symmetry. That is due to Euler’s formula as explained above. Figure 3.16 also shows that the orientation of the PS depends on the orientation of the waves on the original image.

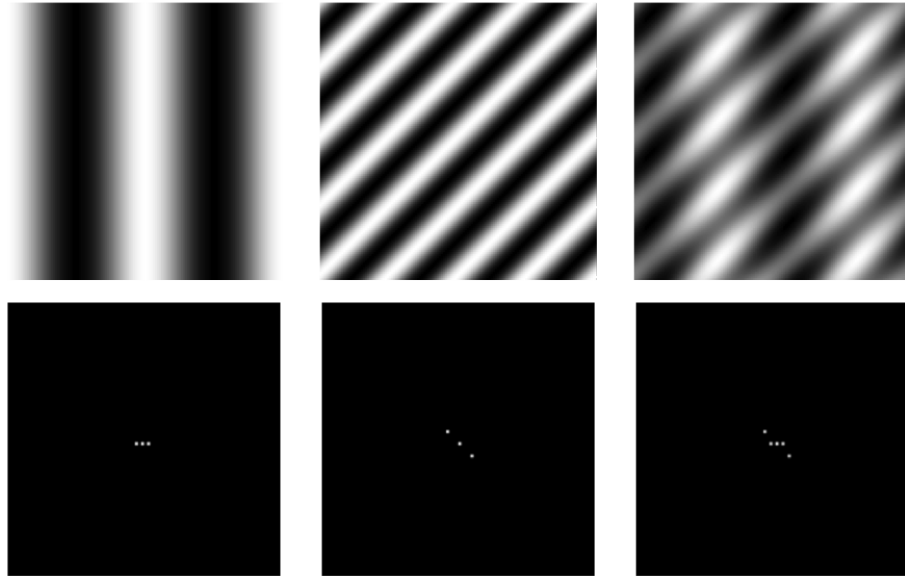


Figure 3.16: Sinusoidal wave images with different frequencies (single or double) and their respective power spectrum. *Images adapted from a website created by Steven Lehar called “An Intuitive Explanation of Fourier Theory” (<http://cns-alumni.bu.edu/~slehar/fourier/fourier.html>).*

If we consider one point of the power spectrum (fig. 3.17), its distance to the center of the PS is representing the frequency of one wave signal composing the original image. The angle of this point (relative to an horizontal left-right oriented line) represents the orientation of this wave signal. Finally the brightness of the point represents the relative intensity of the contribution of this wave to the original image. This brightness is then linked to the brightness of the part of the original image that describes a wave with such frequency and such orientation [64].

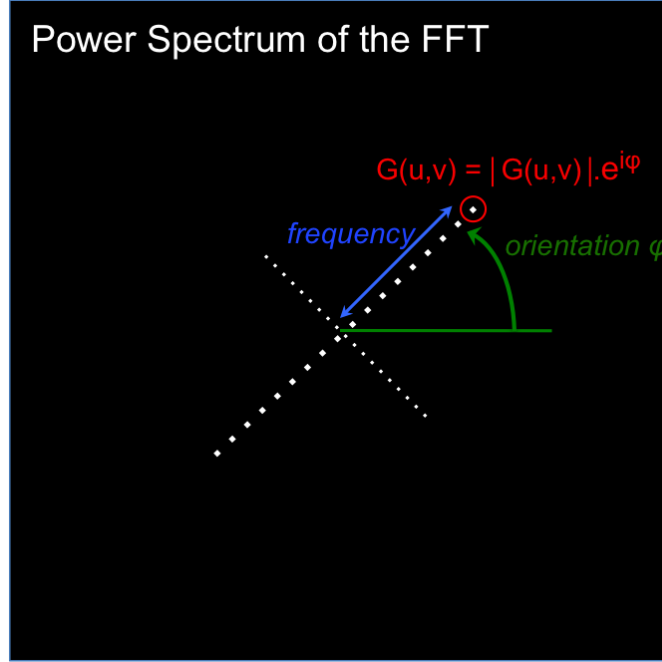


Figure 3.17: Schematic visualization of the information encoded in the power spectrum of the FFT of an image.

The orientation information contained in the FFT of our images is the most important for our study. To evaluate to what degree an image is oriented along a given angle α , we added all the intensities of the PS contained in a double 2D smooth cone (with its apex centered at the center of the PS) centered on α and with an angular aperture 2° . This operation accumulates all the signals of the original image that are oriented along α . By this operation we lose the frequency information contained in the PS. I will discuss later why we chose this strategy and how this method could be improved in the future.

3.3.3 The different steps of the Image Analysis

The steps of the code built and used here are summarized in figure 3.18.

Note: all functions listed below belong to the image analysis toolbox of Matlab (Mathworks, Inc., USA).

Raw Images

The raw images were fluorescent images with different proteins of the cytoskeleton stained. One fluorescent channel shows all cell bodies and neurites (in this case all neurites are marked) by staining of β -III tubulin. Another channel is highlighting the dendrites of the neurons by staining of MAP-2.

Cropping to the largest possible square

Raw images were first cropped to form the largest possible square because 2D FFT requires squared images.

Function used: `imcrop(I,[x,y,height,width])`, where “x” and “y” are the coordinates of the up left corner of the cropping rectangle and “height” and “width” are the height and width of the cropping rectangle.

Subtracting Background

The background of the images were determined by morphological opening (erosion followed by dilation) with a disk-shaped structuring element with a radius of 15 pixels. This operation essentially amounts to a very rough smoothing. The background was then subtracted from the original image.

Function used: `imopen(I,strel('disk',15))`.

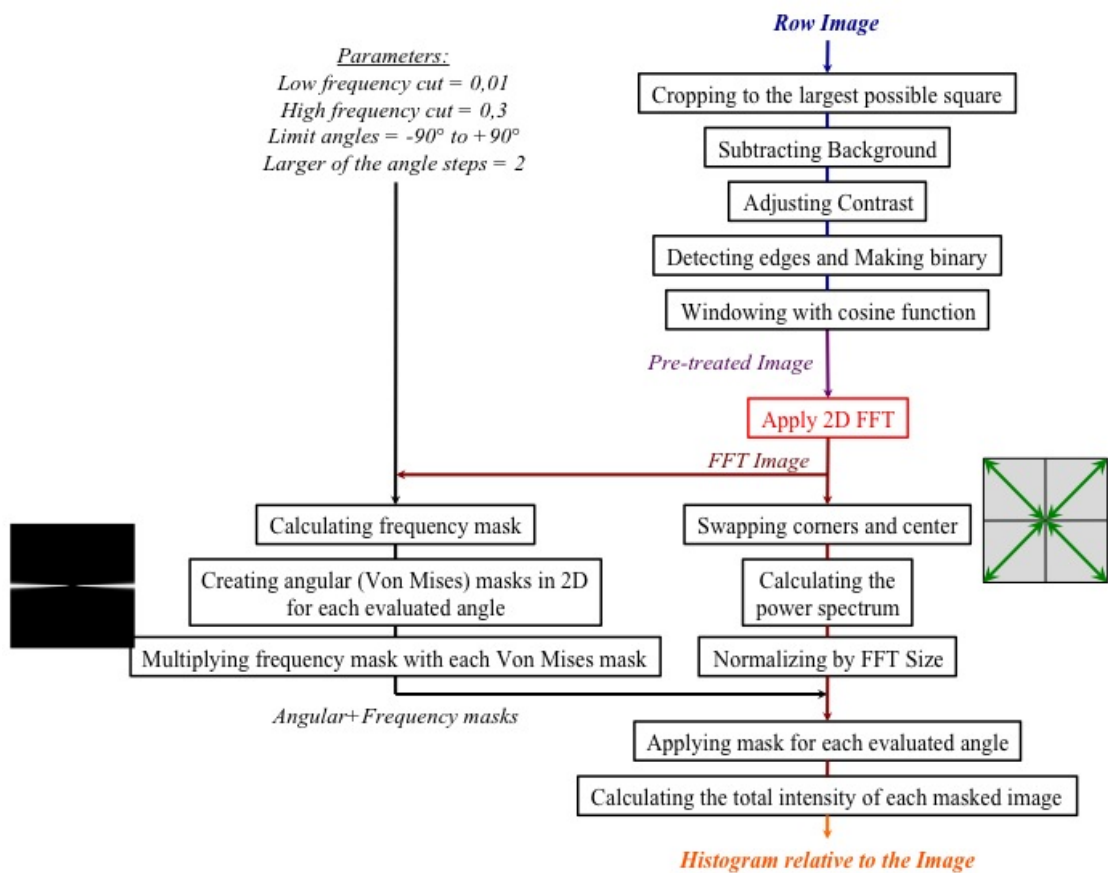


Figure 3.18: Overview of the different steps of the MATLAB program created to assess the orientation of the neurites on fluorescent images.

Adjusting Contrast

Then the intensities of grayscale images were mapped so that 1% of the pixels were saturated at the highest and lowest intensity limits. Two examples of cropped images that underwent background subtraction and contrast adjustment are presented in figure 3.19.

Function used: `imadjust`.

Detecting edges and binarizing

A canny filter was then used to detect the edges (areas of highest intensity gradient) in the grayscale images. This changed the images into binary images in which the only bright pixels delimit the edges of the treated grayscale images. This operation erased most of the cell bodies that were not relevant for our study as we wanted to assess the orientation of the neurites only.

Function used: `edge(I, 'canny')`.

Windowing with cosine function

The windowing was used to smooth the frame edges of the images in order to prevent edge artefacts in the 2D FFT. The FFT of a black squared image show non-zero intensities on the horizontal and vertical centred lines that originate from the two vertical and horizontal edges. Without windowing, the signals at 0° and 90° would always be over-estimated. To prevent this effect edge images were windowed by a function presenting a central symmetry (2D cosine function with a radius equal to $2/3$ of the image size). This way the artefact signal is the same along all the angles so that it does not bias the relative intensities produced by the original image. The result of the edge detection followed by windowing is presented in the middle line of the figure 3.19.

Apply 2D FFT

The Fourier Transform is transforming an image representing space variations of intensities to its equivalent description in the frequency domain. The principle of the 2D Fourier Transform is to decompose an image into a summation of wave patterns with different frequency, amplitude and orientation. If we consider one point of the FFT, its distance to the center of the FFT is representing the frequency of one wave signal composing the original image. The angle of this point (relative to an horizontal left-right oriented line) represents the orientation of this wave signal. Finally the brightness of the point is linked to how much this wave signal is contributing to the original image.

Function used: `fft2()`.

Swapping corners and center

The next step was to swap the corners and center of the FFT. Imagine that you divide the FFT in four squares and then you flip each square so that their central

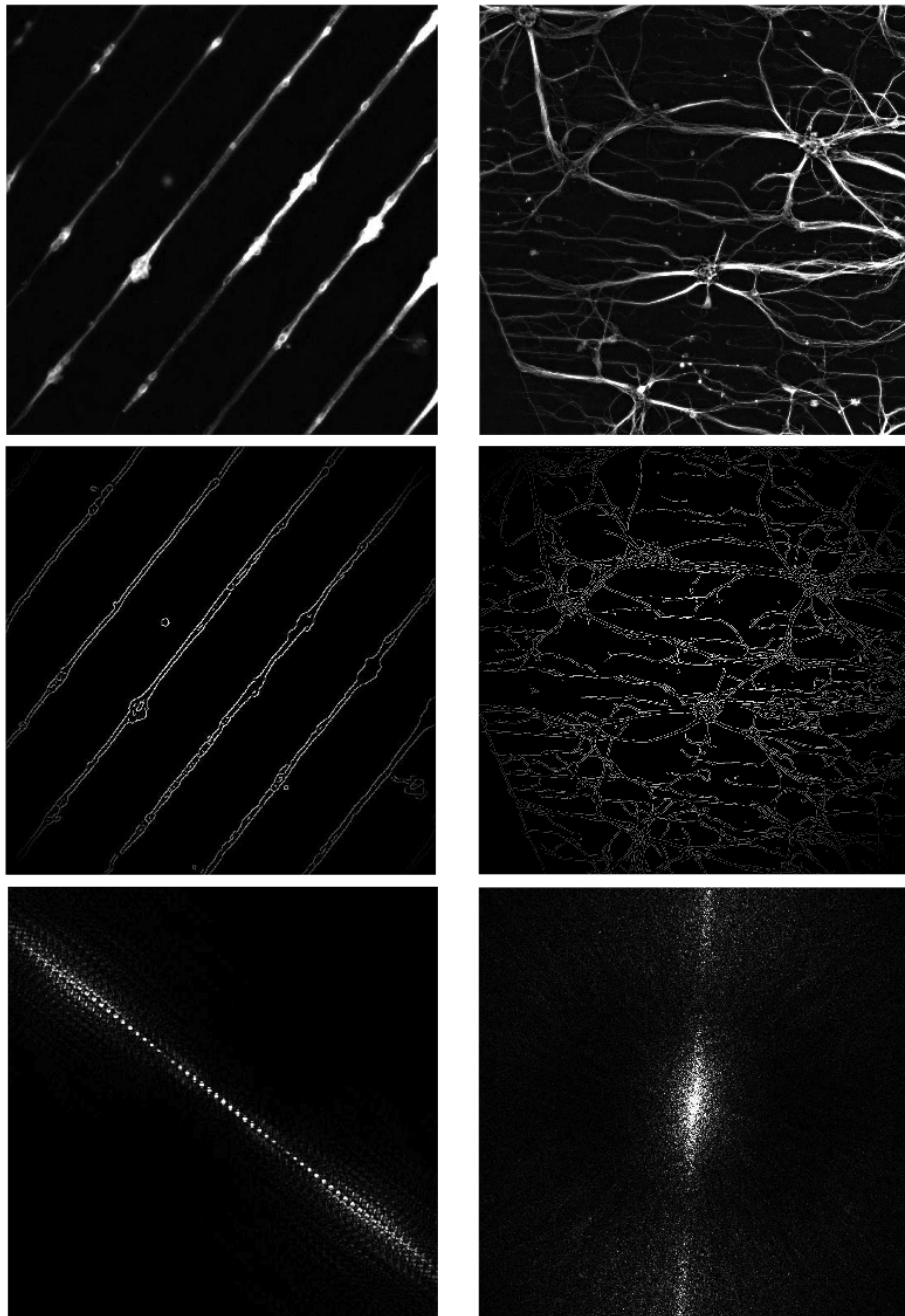


Figure 3.19: Illustration of the main steps of the MATLAB program created to assess the orientation of the neurites. *Example of a uCP-stripe combination image (left column) and a uGro-stripe combination image (right column). Top line: images after background subtraction and contrast adjustment. Medium line: images after edges detection and cosine windowing. Bottom line: FFT of the pre-treated images above.*

corner is now in one of the four outside corners. This operation is used to swap the high and low frequencies of the FFT. After swapping the lowest frequencies of the FFT are in the middle of the FFT, and the highest frequencies are at the periphery of the FFT.

Function used: `fftshift()`.

Calculating the power spectrum

The power spectrum of the FFT is then calculated. For each point the complex number of the FFT is replaced by the square of its modulus.

Normalizing by FFT Size

To normalize the signals according to the size of the images, the power spectrum is divided by the image total area. Illustration of the normalized PS obtained for the two example images are presented in the bottom panels of figure 3.19.

Calculating frequency mask

The angular and frequency masks allowed us to build the angular histograms. First the user choose the percentage of low and high frequency he/she wants to cut from the total frequency distribution. Here we chose to cut the lowest 1% and the highest 30% of the frequencies. This choice was calibrated on a representative sample of 10 pictures. We might be interested in low frequency because sometimes the period of the pattern can be close to the size of the image. On the contrary we are not interested by high frequencies that are mostly associated with the meandering and irregularities of neurites on scales smaller than that of the micro-patterns.

In practice, a matrix of the same size as the PS, with a circular pattern with 0 at the center and a radial increase reaching 1 at the periphery, was created. From this matrix the cut offs were determined and a new matrix was created with 0 for radi smaller than 1% of the maximum radius and for radi larger than 70% of the maximum radius, 1 elsewhere.

Creating angular (Von Mises) masks in 2D for each evaluated angle

Von Mises functions were used to create angular masks. These functions are cone-shaped with smooth edges. The angular aperture of these function was chosen to be around 2° . The formula for the Von Mises is:

$$VM(\theta) = e^{-\sigma \cos(\frac{2\pi \cdot (\theta - \alpha)}{180})}$$

where σ is a constant that regulates the angular aperture of the function, θ and α are angles in degrees. α is the angle on which the function is centered and it variates between -88° to $+90^\circ$ by steps of 2° . The steps were chosen to be of 2° because it was a good compromise between a fine binning of the histogram and a reasonable processing time.

Multiplying frequency mask with each Von Mises mask

Each angular (or Von Mises) mask was multiplied by the frequency mask to obtain what I called an angular+frequency mask. Given the steps that I chose I obtained 90 of these masks selecting directions between -88° and $+90^\circ$ and separated by steps of 2° (where the reference angle or 0° angle is the angle of the micro-pattern). The example of the angular-frequency mask for the angle of 0° is presented in figure 3.20.

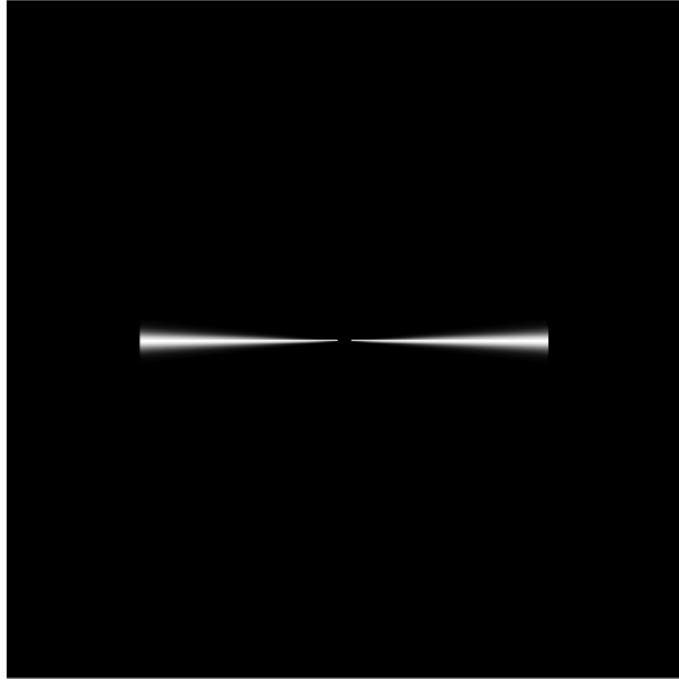


Figure 3.20: Example of the angular+frequency mask obtained for the angle 0° . *The Von Mises function centered on 0° is masked by the frequency mask cutting 1% of the lowest and 30% of the highest frequencies.*

Applying angular+frequency mask for each evaluated angle

Then the power spectrum of the image was masked by each angular+frequency mask.

Calculating the total intensity of each masked image

The total intensity of the power spectrum masked by each angular+frequency mask was calculated by summation of the intensity of each point of these masked images.

Histograms obtained

Finally we obtained for each even angle between -88° to $+90^\circ$ the total intensity

of the power spectrum oriented along this direction. By normalizing the total intensity we obtain the percentage of the power spectrum oriented in each direction. This intensity distribution was then set in relationship to the angle of the micro-pattern, 0° being the angle of the micro-pattern.

3.3.4 Analysis of the highest peak characteristics of the histogram

From the histograms we extracted some parameters for statistical analysis. The Image J plugins used a Gaussian fit on the highest peak to determine the preferred direction and the percentage of the image oriented along this direction. This post-treatment was not very accurate because some of the histograms were noisy. A correlation of the histogram with a $\cos^2$ function centered on the angle of the pattern did not give coherent results. We then decided to extract the main characteristics of the highest peak of the distribution.

Figure 3.21 illustrates the different characteristics extracted. The angle of the highest peak relative to the angle of the pattern was determined and called AngO or “Orientation Angle”. Then the area of the peak between the angles AngO- 14° and AngO+ 14° was determined. It represents the “Dispersion of the orientation” of the neurites around the main orientation.

3.3.5 Routes to explore in order to improve and deepen the orientation analysis method

The method developed proved to be efficient to assess neurite orientation at the population level in dense cultures of neurons. By varying the immunostaining of different cytoskeletal antibodies, one could evaluate the polarization of neurons by measuring the difference in behaviours of dendrites and axons. However, there are many ways to optimize our method and deepen the degree of analysis.

Optimization

The pre-treatments of the images prior to FFT could probably be improved. An edge detection was preferred over a tube-like shape detection, but we did not systematically explore the different types of filters employed for edge detection. For the windowing of the images, we chose one of the simplest possible (a cosine), more complex functions, such as the classic polynomial Welch or Hamming windows, could be considered and tested.

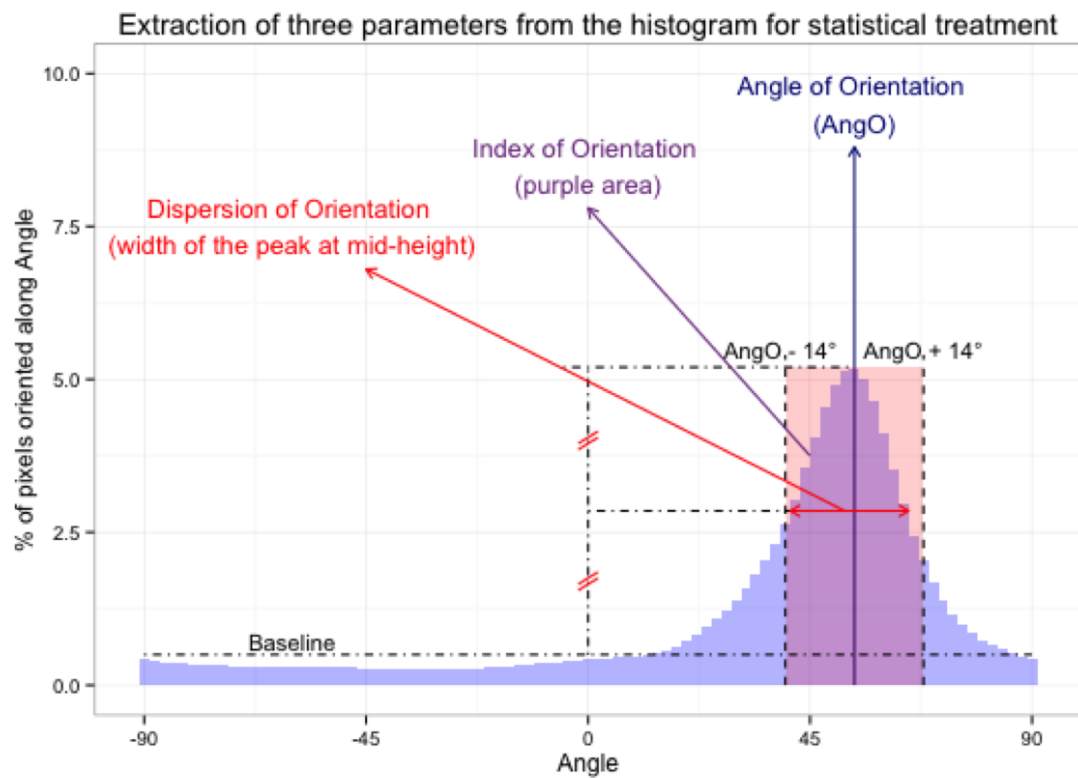


Figure 3.21: Overview of the different parameters (index of orientation, angle of orientation and dispersion of orientation) extracted from the highest peak of the histograms obtained after 2D FFT analysis.

The padding of the images was also explored to a limited degree only. Padding an image means that you increase the size of the image by building a black frame around it. Padding is usually used as an alternative to cropping, in order to obtain square images before applying the FFT. However it can also be used in combination with windowing in order to better reduce edge effects. No padding was used in the presented algorithm. A padding that increased by two the size of the images was tested but did not make any significant difference. Thus, no padding was used in the presented algorithm.

For processing of the power spectrum, better optimized Von Mises function parameters or other masking angular functions might result in less noisy histograms. Initially, simple conical shapes were used but the smoothing by the Von Mises functions raised the signal to noise ratio. The binning of the histogram (the steps of 2°) could be explored in more details. Another method to extract the data from different angles would be to add the intensity of the power spectrum along lines at given angles.

Finally, the parameters extracted from the highest peak of the histogram could potentially be improved. The fixed angular aperture chosen to estimate the area of the peak (28°) could be refined considering the distribution of the width at mid-height of the highest peaks (fig. 3.22). The relatively large aperture chosen might overestimate the orientation of poorly oriented images and thus artificially reducing the difference between micro-patterns and controls.

Using the frequency information from the FFT?

There is information on frequencies in the FFT that we did not use at all. It would be interesting to explore how to use this additional information. It is possible to use for example the frequency pattern along the line of the angle of main orientation. This could allow us to differentiate between thick stripes and thinner stripes (fig 3.23). That would be a way to test the hypothesis that “neurites follow dihedrals”.

Another way of using the frequency information would be to treat separately different range of frequencies. We could separate the frequencies into three categories: (i) low frequencies, (ii) medium frequencies and (iii) high frequencies. Then we could see if the preferred orientation depend on the frequency. That would give us insights on the persistence length of the orientation of the neurites.

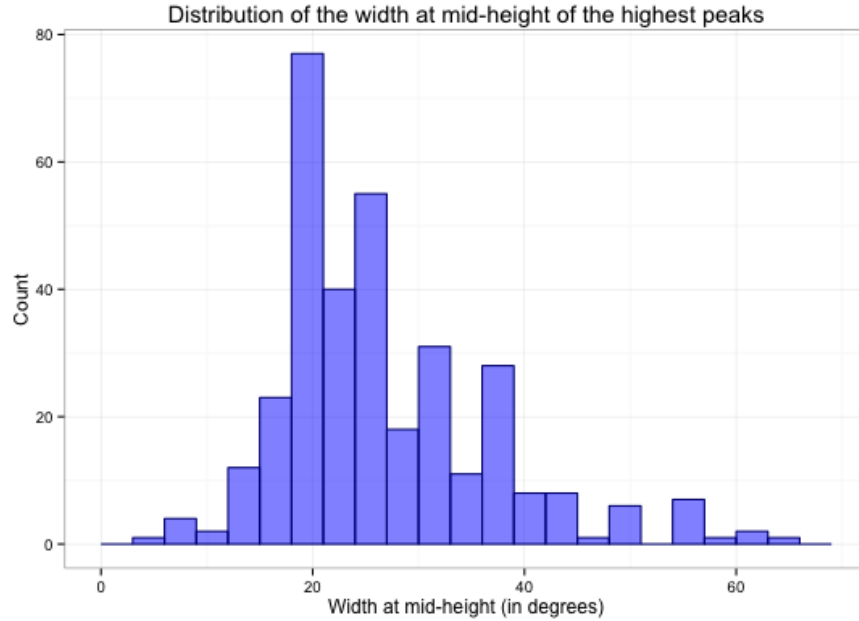


Figure 3.22: Histogram of the distribution of the width at mid-height of the highest peak of the orientation histograms.

Colocalization pre-treatment to observe axonal orientation

To study the differences between axons and dendrites, the analysis done so far on the dataset only gave us the orientation information for either all kind of neurites (β -III tubulin staining) or only the dendrites (MAP-2). Specific axonal markers were not used and are not easy to find, especially for cortical neurons. Nevertheless, gaining access to the orientation for the axons only would be more relevant for our assessment of neuronal polarization. Subtracting a colocalization mask of β -III tubulin and MAP-2 to the β -III tubulin image would have given us this information. Several colocalization algorithm have been tried without success because dendrites have a different apparent diameter on the β -III tubulin and the MAP-2 images, making the colocalization calculation challenging.

Different strategies

Although the method employed to assess the orientation of the neurons was proven quite powerful, we could try other methods to assess the orientation of the neurites. Auto-correlation functions of the FFT could be used to test the repetitiveness and/or directionality of images.

For a study of patterns of connectivity in cultured neurons, Renaud Renault recently developed another method to evaluate neurites orientation. His method is

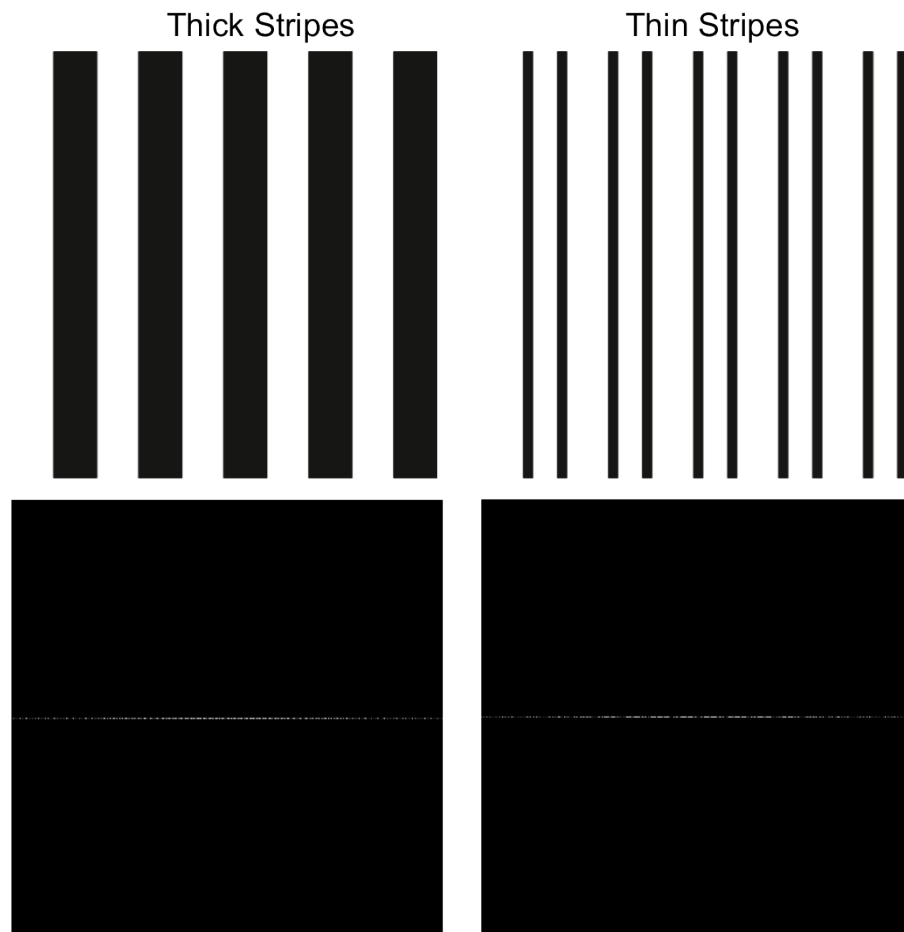


Figure 3.23: Examples of thick and thin stripes pattern and their power spectrum. There is a difference in the frequency pattern in the power spectrum that is not detectable with the method employed so far.

based on the correlation between the original image and elongated scanning wavelets with different widths and all possible orientations. This method has a longer processing time but is more direct and should provide direct information about the persistence length of the orientation of the neurites. This method could be compared with the one I used here.

It would be also worth using classical neurite tracing methods on some of the images and compare quantitatively the results of the two methods. This would allow us to share our method, present its main advantages and limitations and explain in which cases it is more powerful than the classical method used.

3.4 Further discussions and perspectives

Beyond the method used and its possible improvements, it is interesting to discuss the dataset used, the results obtained and the hypothesis that still need to be formally demonstrated by future experiments. I will now discuss the points that I find the most important.

3.4.1 Analysis of other parameters on the orientation and polarization of neurons

In the sections above, only one part of the dataset was presented. The information collected and extracted from the images were much richer. I tested the influence of the density of the culture and of the neuronal subtype on the index of orientation of the neurites.

Influence of the neuronal density on the orientation

To our surprise we could not observe a significant influence of the density of the culture on the orientation of the neurites.

This is counter-intuitive because neurons attract each other through the secretion of growth factors. However, it is important to note that the densities measured here are local because they result from counting cell nuclei on the images. The concentration of neurons at seeding was supposed to be the same in all experiments.

The dispersion of the local density of neurons for a unique seeding concentration highlight the fact that the seeding is not homogeneous among or even within chips. A better control of the flows would probably decrease these inhomogeneities.

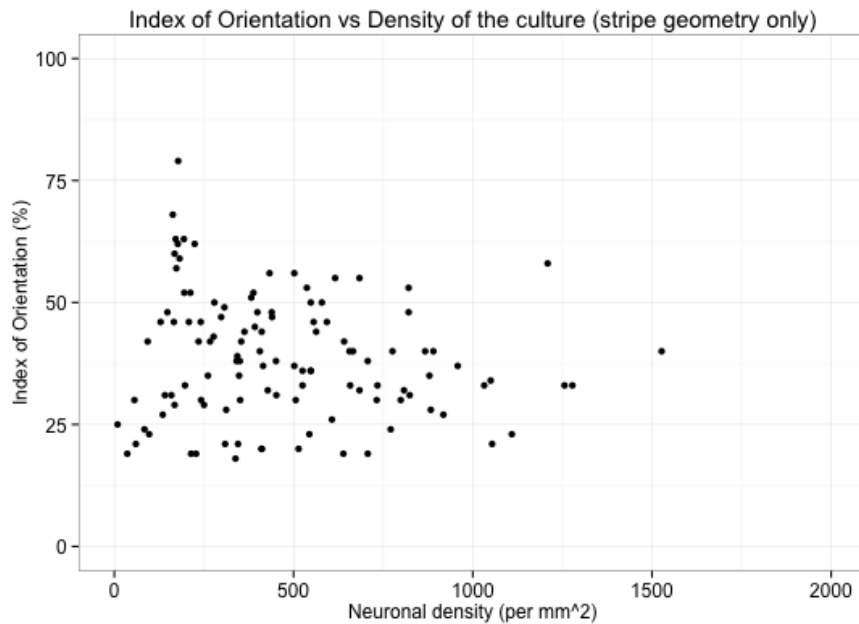


Figure 3.24: Variation of the index of orientation depending on the local density of neurons.

Influence of the neuronal type on the orientation

I also looked for the influence of the three neuronal subtypes cultured in the experiments: Cortex, Hippocampus (mostly Ammon's Horn) and Striatum. The differences are interesting. For the stripe geometry the only significant difference is observed for the dendrites of the hippocampus which are more oriented than the dendrites of cortex or striatum (fig. 3.25). This result highlights the importance of separately exploring axons and dendrites, and also calls for a careful consideration of neuronal subtype.

As I will discuss in the next section, some biases remained in the dataset, due to experimental difficulties and to the impossibility to fully equilibrate it during the time of the thesis. Due to this bias, we could not conclude on the differences observed between the different neuronal subtypes.

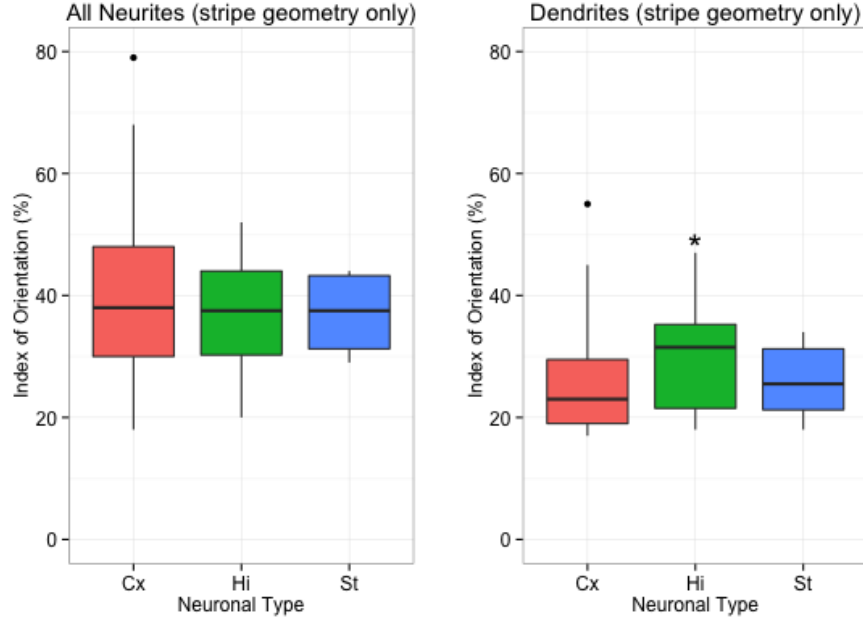


Figure 3.25: Variation of the index of orientation depending on the neuronal type and neurite type (* for p-value < 0.1).

3.4.2 Limitations of the dataset used so far and future experiments

A biased dataset

The dataset used comprises six experiments. From these experiments, more than 700 images were analysed. The biased repartition of these images over the different techniques and geometries is presented in figure 3.26.

Another bias can be observed in the repartition of the width and gap size for the different geometries and is presented in figure 3.27. In fact, the width and gap of the stripe geometry were diverse. On the contrary the distribution of width and gap for the square and circle geometries was narrow. More sizes should be explored in these cases.

Despite these biases, statistical analysis could still be done with care and the results presented in section 2 of this chapter are reliable.

To verify that we could compare the 6 experiments we checked for differences in the controls of these experiments and did not find any significant difference (fig 3.28).

		Numbers of images Analysed
Technique	uCP	123
	uGro	292
	iMP	192
Geometry	Stripe	244
	Control	127
	Square	44
	Circle	65
	Fold Control	127
Neurites Type	All Neurites	336
	Dendrites	271
Neurons Type	Cortex	494
	Hippocampus	79
	Striatum	34
TOTAL		715

Figure 3.26: Table of the repartition of the images of the dataset over the different techniques, geometries, neurites type and neuronal subtypes.

I also tested whether the magnification of the images (10X or 40X) or the cropping of images that presented initially multiple geometries had an influence on the index of orientation. Only the control geometry was again tested. Unfortunately, between cropped and non-cropped images, and between 10X and 40X magnification images, the differences were small but significant (fig. 3.29).

These significant differences are probably limiting the significance of the results presented in section 2 and it will be important in future experiment to stick to one magnification and to not take pictures presenting different geometries. However, the differences observed were small and because the repartition between 10X and 40X images between the different techniques and geometries was relatively balanced, I do not think that it could invalidate or reverse the results above. In addition, These differences could be corrected by more complex statistical analysis.

Future experiments

As it was just mentioned, more experiments need to be done to confirm the results presented in section 2. It will be also interesting to explore further some of the hypothesis that arise from this first dataset.

Clarifying the role of the width vs the gap in the orientation of neurites.

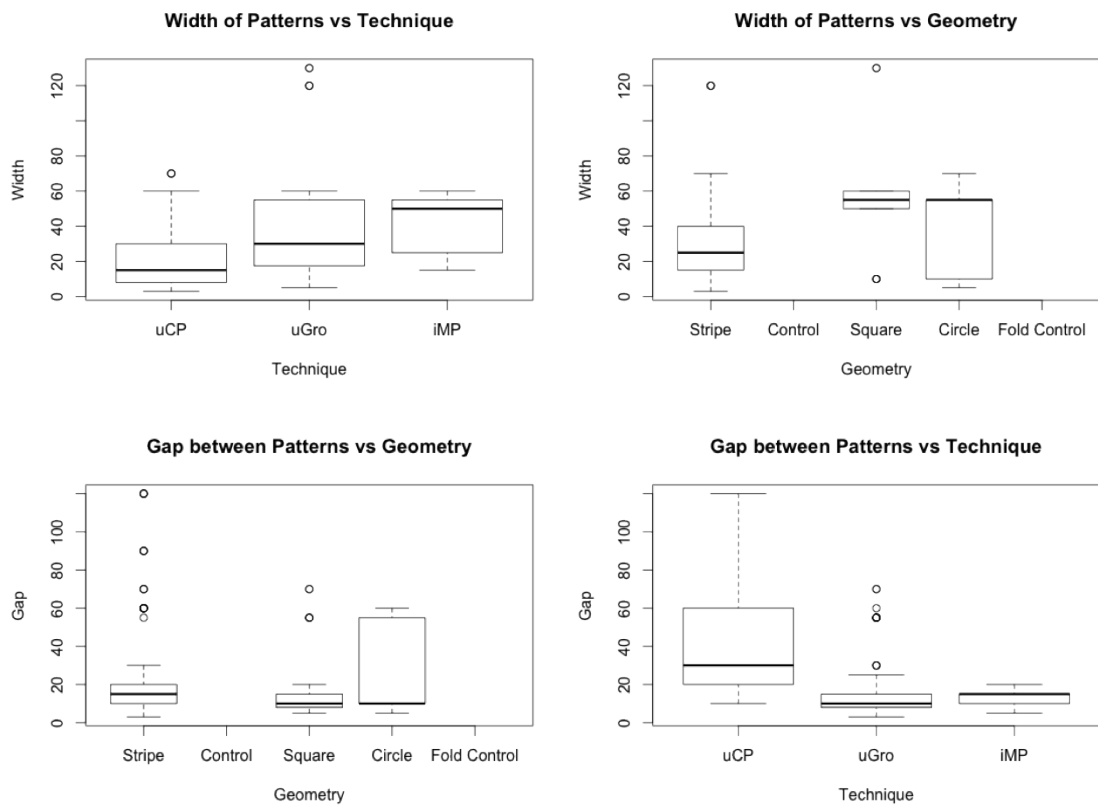


Figure 3.27: Illustration of the biased repartition of width and gap size for the different geometries.

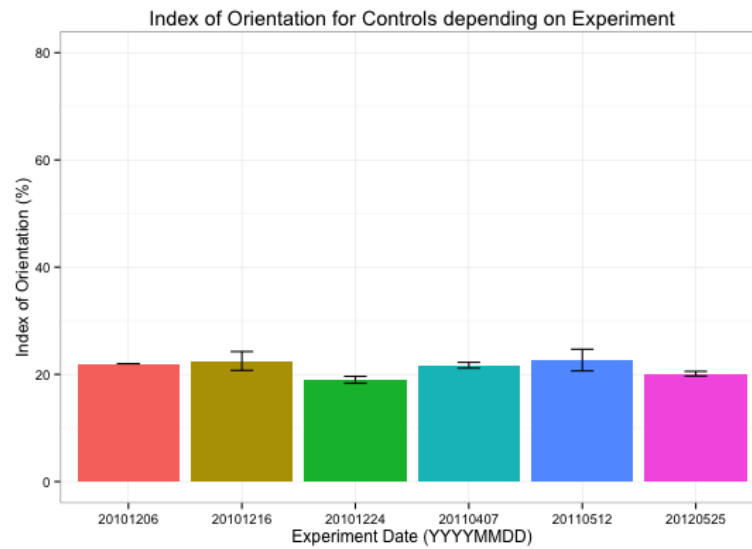


Figure 3.28: Comparison between the index of orientation of the controls of the 6 experiments of the dataset.

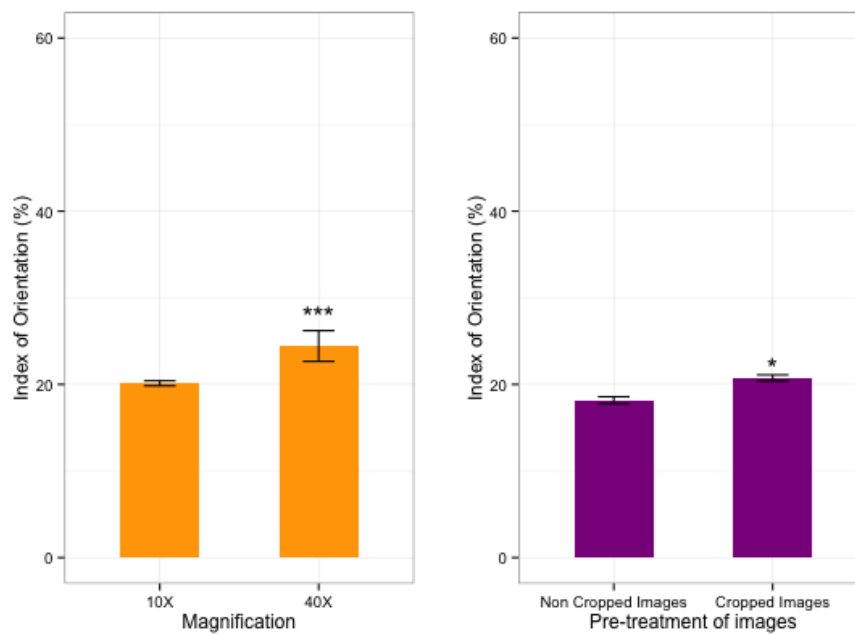


Figure 3.29: Comparison between the index of orientation of images with different magnification or images that were cropped prior to the analysis because they presented initially different geometries (*** for p-value < 0.001).

We observed variation in the efficiency and specificity of capturing cell bodies as well as in the index of orientation of the neurites depending on the ratio width/gap. It will be interesting to separate the influence of these two size parameters to be able to optimize future design for building neuronal networks in our chips.

Clarifying the differences between techniques.

Even though the differences between techniques were most of the time limited, in the stripe geometry, we can observe that iMP yields better orientation than uCP, followed by uGro. It is even clearer if we look at figure 3.8 in which the stripes are sorted by their dimensions. This tendency supports the intuition that adhesive patterns orient more the neurites than pure mechanical constraints and that the combination of two constraints (chemical and mechanical) in the case of iMP are better than adhesive patterns alone. As we discussed in section 2, this intuition need to be balanced depending on the width and gap of the stripes. Nevertheless, it would be interesting to have more and better data to further strengthen this study.

Further studies on the confinement of neurites.

The confinement of neurons inside the patterns has not been analysed very extensively, although it was considered in some specific situations. In other words, the question is: are the neurons completely trapped in the micro-patterns, or can they escape from them? In some cases this notion was approached. First when we observed that neurites can cross over short non-adhesive distances in the uCP-circle combination. Second when we observed that neurites are mostly growing along the bottom of the walls of the grooves. But the confinement of the neurites inside the patterns was not quantified properly.

One way to quantify this confinement is to mask the images according to the patterns, selecting either the inner part of the patterns (adhesive part or bottom part of the grooves) or selecting the outer part of the patterns. We could then compare the total intensity of these two selections and assess which percentage of the culture is inside the patterns vs outside the patterns. Even though the idea is quite simple, in practice, it is not so easy to create the masks, especially in the case of uGro pattern in which we cannot color the inner part of the patterns but can just detect the edges of the patterns on translight images.

Maybe the frequency information of the FFT could be exploited in this direction as well. This confinement will need to be quantified as it is particularly important for the polarization of the neurons. If neurites are too much confined there is no chance to orient the axons and dendrites in different directions. This is one of the reasons why more permissive techniques such as uGro are not necessarily worse than

iMP, it all depends on the application thought after. Finally, the hypothesis that neurites confined in micro-grooves could resist to higher shear stress would also be interesting to study.

Further studies on the “neurites follow dihedrals” hypothesis.

The hypothesis that neurites follow dihedrals (or follow the bottom of the walls in micro-grooves) needs to be further evaluated in two ways. First, it would be good to find a way to quantify better the position of the neurites on the images. This could be done by masking the edges of the patterns and evaluating how much of the total intensity of the image is in these regions. Exploiting the frequency information of the FFT could also give insight on that part.

Second, new experiments need to be designed to test this hypothesis thoroughly. It would be interesting to test the influence of the depth of the micro-grooves on the orientation of the neurites. Another interesting experiment would be to build surfaces with dihedrals that would not be embedded in grooves, basically just steps with different geometries and see how it affects the orientation of the neurites.

Finally the uCP-square combination needs to be explored. This experiment will constitute a negative control for the two assumptions (1) that neurites follow dihedrals and (2) that neurites cross the shortest non-adhesive distances. For this kind of micro-patterns the orientation of neurites should be close to the control condition. These patterns do not provide any structures so the neurites should not be concentrated at the edges of the patterns. In addition, the gap between two adjacent squares is constant so the neurites should cross indifferently in all directions. This last point should however depend on the absolute distance between the squares.

Dynamic studies

Finally to further evaluate if neurites follow dihedrals and more generally to explain some of the results presented in this chapter, it would be interesting to do some live experiment to see how axons and dendrites develop in 3D environments we are designing to guide them.

For this purpose, two lentiviruses staining respectively the tubulin and the actin of neurons have been bought and tested successfully. Some neurons have been transfected by the two viruses. We can then follow in live how the neurites develop and we could adapt the image analysis method to assess the orientation of the neurites at different developmental stages *in vitro*.

3.4.3 Using our results to design neuronal networks in microfluidic chips

The results presented here can be used to design micro-patterns associated with macro-channels to build a three-compartmented or even four-compartmented neuronal network mimicking the perforant pathway. The perpendicular design of the macro-channels presented in the chapter 3 would be conserved. As long as we do not need single neuron positioning, I would use microfluidic flows and co-flows to seed the different populations. To prevent undesired synaptic connections I suggest using the micro-groove technique. I would try to find the optimal conditions for which the axons would be fully guided by the grooves and the dendrites could still oscillate between grooves. Different depth of grooves could eventually be used in different parts of the chip. Deeper grooves (around $12 - 15\ \mu\text{m}$) were already tested and seem to completely confine all neurites. This behaviour could be utilized to build non-crossable barriers for neurites in strategic positions.

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Chapter 4

Towards Cognitive Processes On Chips

4.1 Our approach and challenges

The following project was motivated by the interest of the In Vitro Artificial Intelligence Club of FDV doctoral school, founded by Jeremie Sibille in 2008, that I joined at the beginning of my PhD. Alternating between invited talks, journal clubs and brainstorming sessions the club is gathering graduate, master and PhD students. Our objective was to better understand learning processes by reproducing them *in vitro*. Last year the IVAI club joined Fabelier (parisian Fab Lab, founded by Antoine Mazières) and our interest extended towards brain-machine interface and robots.

In the IVAI club I met Renaud Renault who joined Curie's Institute to make an M2 internship with me. Inspired by the club ideas Renaud had the original idea to try to build a living perceptron *in vitro*. In computer science, a perceptron is classically a structured artificial neuronal network that can learn how to classify inputs into several possible categories of outputs [1, 2]. The idea was then to build a structured neuronal network *in vitro* and try to teach the network to sort inputs into different outputs. More precisely, the idea was to build a network with three populations of neurons as inputs and two populations of neurons as outputs. In principle, as illustrated in figure 4.1 this network could then be trained so that for a given input signal (for example, two input populations firing and the third resting), the network would give a predicted answer through the two populations of neurons

of the output layer (for example, one given output population firing and the other resting).

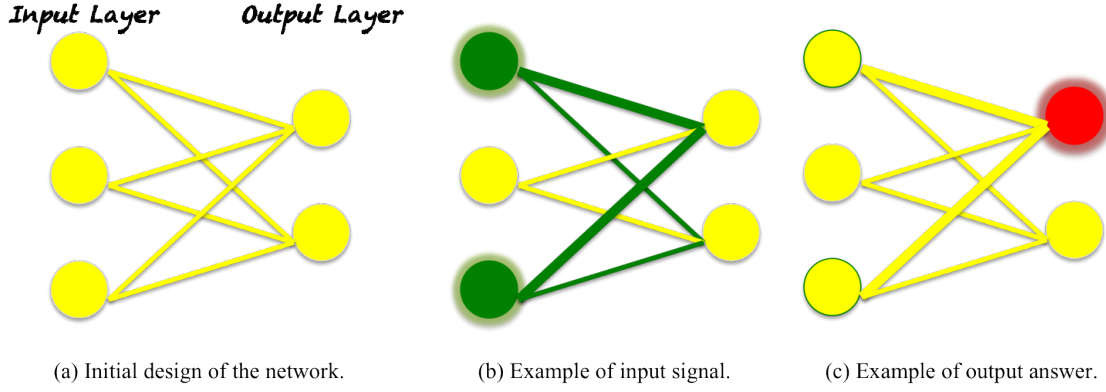


Figure 4.1: Illustration of the principle of the living perceptron envisioned by Renaud.

I mastered all the techniques required to build the structured and oriented neuronal network envisioned. Live calcium imaging could also be used to record the activity of the network. The challenge was then to find an easy way to be able to excite selectively sub-populations of the neuronal network. Micro-electrode arrays (MEAs) are usually a great tool to excite and/or record neuronal activity but we did not have access to such technologies, and anyway it is not trivial to couple MEAs with microfluidic chips. We were also both convinced that it was worth trying to build methods as simple as possible (going towards do-it-yourself methods).

Inspired by *in vivo* optogenetic techniques, we decided to develop a fully optical method to excite and record sub-populations of neurons in oriented *in vitro* networks. The following section consist in the draft of a publication describing this method and was co-written with Renaud.

4.2 Interfacing Neural Devices: a Convenient and Fully Optical Method

Note: This section is a publication in preparation for submission. This section was written by Renaud Renault and myself.

Authors: Renaud Renault [‡], Maéva Vignes [‡], Jean-Louis Viovy, Samuel Bottani.

4.2.1 Abstract

Electrodes are commonly used to excite and monitor neurons, including in the context of *in vitro* neuronal cultures. Here, we offer a new way to simultaneously excite and monitor the activity of neurons by purely optical means, by combining readily available optogenetic techniques and calcium imaging. We show that Channelrhodopsin-2 (ChR2) and Calcium Orange™ indicator spectra are compatible to excite and record neuronal culture activity. LEDs allow us to induce bursts in cultures transfected with ChR2 and in turn, the Calcium Orange™ signal allow us to monitor large scale neuronal responses without interfering with the (optogenetic) stimulations. We used this dual method to infer functional connectivity inside microfluidic culture devices, where two neuronal populations were asymmetrically connected through funnel-shaped microchannels. We propose optical fibers combined with microfabrication as a convenient tool to increase the spatial resolution of the excitation, by allowing the experimentator to target and excite a subpart of reconstructed *in vitro* neuronal network.

Keywords: Calcium Imaging, Optogenetics, Microfluidics, Neuronal Networks, In Vitro.

4.2.2 Introduction

Behind the apparent simplicity of dissociated neuronal cultures lies an incredibly rich catalogue of behaviours present *in vivo*, including growth and differentiation [3, 4], plasticity [5, 6], electrical activity [7–10] and information processing [11–13]. The cultures of neurons, extracted from healthy or diseased animals, have thus become rapidly an invaluable tool to understand the nervous system, from the molecules involved in neuronal functions, to the behaviors produced by embodied cultures. Recently, neuronal cultures have been enriched by new nano- and micro-technologies that have allowed neuronal networks to be reconstructed with oriented synaptic connections [14–19]. These oriented neuronal networks are opening new routes for studying cognitive processes or neuronal disorders *in vitro*.

The fact that we can stimulate and record the culture behaviour is promising to understand neuronal computation, the effects of external stimulations during development, neuronal plasticity, and learning. We have a myriad of methods to stimulate and record neuronal networks *in vivo* [20–23]; however, we still need to develop methods for *in vitro* applications.

Microelectrodes Arrays (MEAs) have first emerged as a choice tool to stimulate and record neuronal culture activity; they have fast temporal resolution and extended spatial resolution, and their non invasiveness makes them particularly suited for long-term experiments. However, MEAs also have limitations; for instance, it is difficult to use them beyond the context of simple two dimensional cultures. It is

also worth noting that the costs and technical efforts necessary to set up a MEA platform are substantial, and might not be adapted to a laboratory that would only need MEAs sporadically.

Electrodes can be completely circumvented by using optical methods to excite and record neuronal activity both *in vivo* and *in vitro* [24–28]. Recently, the field of optogenetics has developed alternative methods to excite neuronal activity, including the transfection of genes coding for light-gated ion channels into neurons [29]. Depending on their nature and the associated ionic flux, photo-activation of the channels depolarizes or hyperpolarizes the membrane, and ultimately induces or inhibits action potentials in the modified neurons. In addition, structured light can be used to excite locally targeted sub-populations of neurons [30,31]. Action potentials in neurons go along with a calcium influx, it is possible to monitor indirectly the activity of neurons containing calcium indicators using a simple fluorescence microscope. Calcium indicators are fluorescent dyes which spectra change upon binding calcium ions.

However, the use of light to concurrently stimulate and monitor neuronal activity requires compatible wavelengths in order to avoid constantly stimulating the background of the cultures during observation. Taken apart, Channelrhodopsin-2 (ChR2) variants and Fluo-4 are the most efficient tools for optogenetic stimulation and calcium imaging, respectively [32,33], but the two are not compatible according to the aforementioned criterion (fig. 4.2). There is not much choice regarding the wavelength used in optogenetics, but fortunately, several other calcium indicators exist.

In this paper, we show that Calcium Orange™ indicator, which spectrum is red-shifted compared to Fluo-4, is a suited tool to record activity of neuronal culture transfected with ChR2. Previous *in vivo* studies have already shown this compatibility [27,28]; however it is the first time that this compatibility is characterized *in vitro*.

We demonstrate the usefulness of combining the two technics by inducing - with LEDs - and recording large scale bursting events to evaluate functional connectivity in neuronal cultures. Finally we investigate the possibility to use optical fibers coupled to LEDs to increase the spatial resolution, which allows us to control the neuronal activity within microfluidic chips, where the use of electrodes is difficult.

4.2.3 Material and Methods

Microchips

The microchips consisted in two parallel channels (1 mm width $\times$ 55 μ m height) either insulated or connected through an array of funnel-like micro-channels [17] (15 – 3 μ m width $\times$ 4 μ m height). These micro-channels function as “axon-diodes”. They allow axons to grow only in one direction and block them in the opposite direction. This special design allowed us to reconstruct oriented synaptic connections

between two populations of cortical neurons. The PDMS microchips were assembled to glass cover slips No.1 using plasma bounding, filled with Poly-D-Lysine (PDL) at 10 $\mu\text{g}/\text{mL}$ and incubated overnight at 37°C to coat the inner surface of the channels. The microchips were rinsed with DMEM before the cells were seeded.

Note: for more details refer to chapter 2, section 2.2.1 on page 35.

Neuronal cell culture

Primary cultures were done using mice cortical neurons extracted and dissociated from E17 embryonic brains. Timed pregnant wild-type OF1 mice were directly provided by Charles River. Dissociated cortical neurons were seeded in the microchip at a concentration of $45 \cdot 10^6$ neurons/mL and incubated a few minutes at RT to allow neurons to attach to the PDL substrate. Complete medium (DMEM 10% FBS with B27 and N2 supplements) was finally added and the cultures were placed into a 37°C incubator with 5% CO_2 .

Viral infection

A lentivirus construct was used to transfect the SYNI-ChR2-YFP cassette into the neurons [29]. Cortical cultures were infected a few hours after seeding using diluted solutions of lentivirus ($\sim 10^6$ TU/ml) to yield 20% transduction rate. The microfluidic chambers were emptied, then viral dilutions were added, allowed to sit overnight at 37°C, and finally replaced by fresh medium. The experiments were performed from 5 days after infection on.

Calcium imaging

The 50 μg of Calcium Orange™ (Molecular Probe® Inc., USA) in each tube were resuspended into 10 μL of anhydrous DMSO to yield ~ 4 mM stock solutions. Calcium Orange™ stock solutions were diluted into filtered Recording Buffer (NaCl 129mM, KCl 4mM, MgCl_2 1mM, CaCl_2 2mM, Glucose 10mM, HEPES 10mM) to obtain a loading solution with a final concentration in calcium indicator of 10 μM . The complete medium in neuronal cultures was replaced with loading solution and the cultures were incubated at room temperature for 40 minutes in the dark. The loading solution was then removed and replaced by DMEM 0%SVF + B27, and the cultures were allowed to recover at 37°C for 30 min in the dark before imaging. To monitor the neuronal activity, a Nikon Eclipse-Ti inverted fluorescence microscope was used with standard Cy3 filters.

Stimulation

Optical stimulations were performed with Nichia Blue LED (model: NSPB300B). The LEDs were driven with an Arduino Uno board in order to generate reproducible

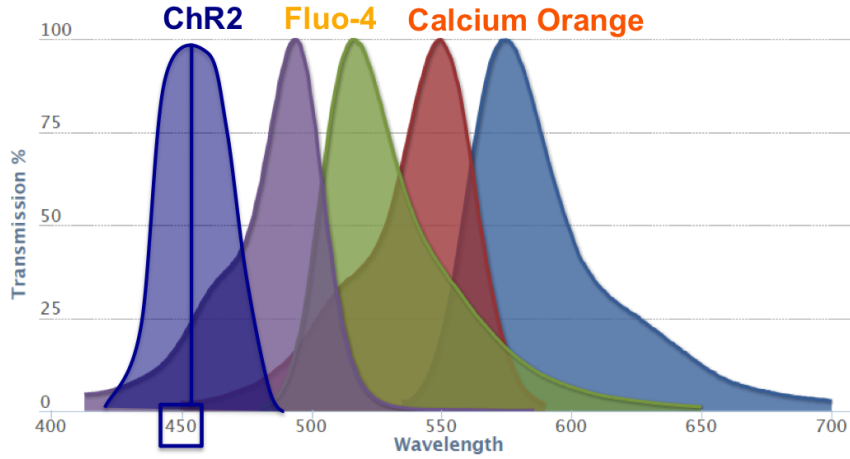


Figure 4.2: Representation of the absorption and emission spectra of Fluo-4, Calcium-Orange and channelrhodopsin. *Image modified from www.curvomatic.com.*

stimulations. For global stimulation, a LED was mounted on the side of the microscope's objective and the beam was superimposed with the field of view, without any additional optical device. For spatially controlled stimulations we used 1mm PMMA optical fibers. The diameter of the fiber at one end was reduced to $\sim 500 \mu\text{m}$ by stretching the fiber in boiling water steam. The two ends were sanded with a rotary tool to make plane interfaces. The large end of the optical fiber was coupled to the LED using two convex lenses, and the narrow end was illuminating the field of view, at a distance of $\sim 1 \text{ mm}$ from the glass cover slips on which rest the neurons, and with an incident angle of 45° , so that the fiber does not sit in the optical path of the light during imaging.

Analysis of calcium imaging traces

Raw fluorescence traces (fig. 4.3(a)) were first cleaned from the interference coming from the scattering of the excitatory blue light. The amount of light scattered was evaluated concurrently to the recording of neuronal activity in an area devoid of neurons (fig. 4.3(b)) and subtracted from the associated calcium trace up to a multiplicative factor that was determined independently for each stimulation to maximize the smoothness of the cleaned trace (that is minimize the integral of the derivative squared). Because the resulting traces were still altered by noise and eventually photobleaching (fig. 4.3(c)) we used a second filter to homogenize the results. This filtering consisted in convolving the fluorescence trace with an a function equal to -1 on $[-0.5, 0[$ seconds, 1 on $[0, 0.5[$ seconds and 0 otherwise. The resulting trace retains the information about the presence and size of the bursts while eliminating the noise and other fluorescence changes that occur at different time scale than the convolving function (fig. 4.3(d)).

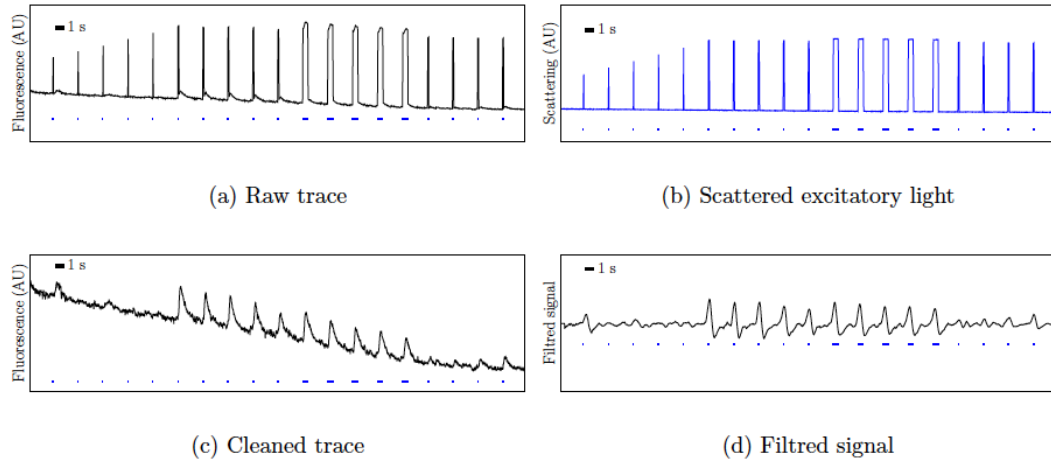


Figure 4.3: Processing of fluorescent trace.

Note: some of the traces presented in the following sections were corrected for photobleaching but were not filtered. As shown on figure 4.3(d), filtered peaks have a tail under the baseline. The shape of the peaks is then looking like an action potential shape. That could be misleading because we are not looking at single action potentials here but rather at population bursts. The filtering may have to be changed to a correction of the photobleaching only prior to publication, but it has not been done on all traces so far.

4.2.4 Results and Discussion

First, we observed the calcium imaging traces of single populations of neurons transfected vs non-transfected. Then we used chips comprising “axon-diodes” and we tested the transmission of our induced bursting either in the passing direction of the diodes or in the blocking direction of the diodes. In this two first set of experiments, the stimulation was directly done by blue LEDs positioned underneath the seeded macro-channels. Finally we explored the excitation of a sub-population of neurons in the same macro-channels using optical fibers to restrict the area of the blue light stimulation.

ChR2 and Calcium Orange™ are compatible

The figure 4.4 show the result of the stimulation of non-transfected vs transfected neuronal populations. The trace for the non-transfected population show some peaks at the stimulation time that might be due to residual light from the stimulation itself. However these peaks are small compared to the ones observed for the transfected population. The highest peak of the non-transfected trace is decorrelated from blue light excitation. Thus, the green light used during calcium imaging

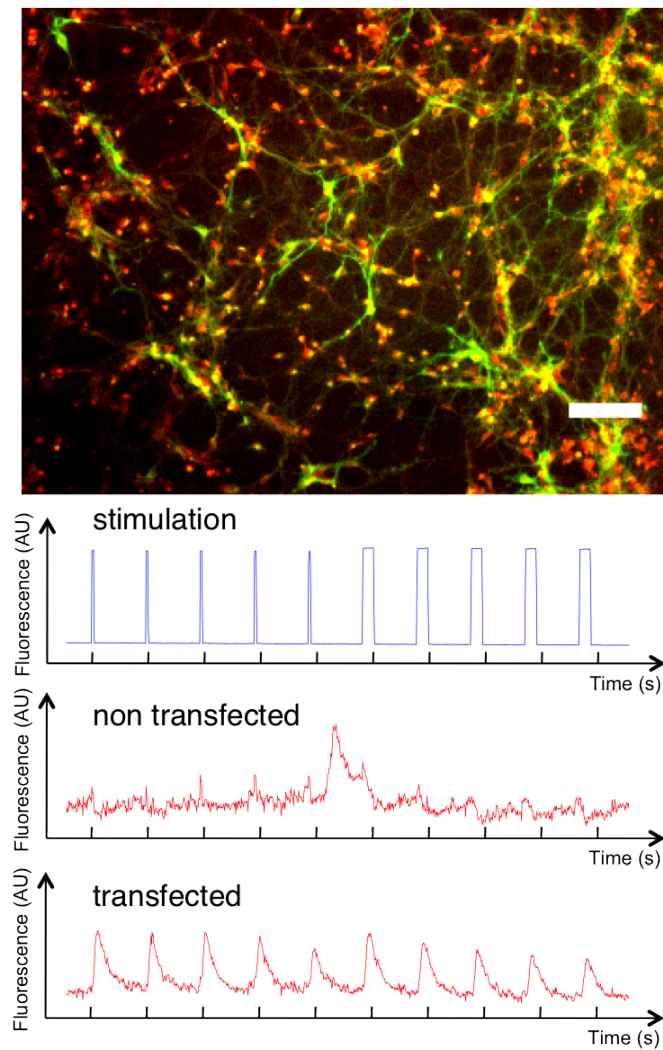


Figure 4.4: Basal and induced activity in neuronal cultures observed with Calcium-Orange (DIV10). *Picture* : merged fluorescence signal of ChR2-YFP (green channel) and Calcium-Orange (red channel) in a transfected culture (scale bar 100 μm). *Traces* (from top to bottom): stimulation pattern, activity in a non-transfected culture under induction, and induced activity in a transfected culture (stimulations 125 or 625 ms).

with Calcium-Orange™ seems to have no significant effect on the spontaneous activity of transfected cultures, as opposed to what we expect with the Fluo-4 indicator.

The LED blue light used for stimulation solely induced the bursting events (fig. 4.4, bottom trace). The efficiency of burst induction was dependent on the duration of each stimulation. Under our experimental condition, the minimal duration of stimulation able to induce burst was 25 ms but only 125 ms stimulations could induce bursts reliably, with a 87% efficiency at DIV14 ($n=54$ stimulations, 7 different cultures). Using longer stimulations of 625 ms did not cause significant improvement (efficiency 90%, $n=62$, 7 different cultures) but caused a subsequent desensitization period during which the efficiency of 125 ms was reduced to 54% ($n=26$, 6 different cultures) along with a decrease of the amplitude of the bursts ; it is not known at this stage whether Chr2 is involved in this desensitization or if it is rather an intrinsic property of cultured networks homeostasis.

The efficiency of burst induction was positively correlated with the day post infection and the transfection rate of the culture; it could also substantially be increased by using dedicated powerful light sources, instead of off-the-shelf components, to allow shorter and more effective stimulations.

Deciphering functional connectivity in artificial networks

The aforementioned combination of optogenetics and calcium imaging methods was used to monitor the responses of large neuronal assemblies upon stimulation, and detect the presence of anisotropic functional connectivity in artificial networks consisting of two populations connected through “axon-diodes”.

Only one of the two neuronal population was transfected with Chr2, allowing spatial control of bursting initiation, while the activities of both populations were recorded simultaneously (fig. 4.5). For DIV6 cultures, induced bursting events were completely confined to the transfected populations independently from the orientation of the axon diodes ($n \geq 10$ stimulations in 4 circuits, for either condition), suggesting that functional synapses between the two populations were not sufficiently developed at this stage even with axon diodes in the forward orientation (from transfected to non transfected population). At DIV14, the bursting events induced in the transfected population only spread to the non-transfected population when the diodes were in the forward direction (fig. 4.5).

Our result shows that the functional connectivity between the two neuronal populations, as revealed by the concomitant use of optogenetics and calcium imaging, is consistent with the unidirectional structural connectivity induced by the axon diodes. This result are in agreement with Peyrin et al. who demonstrated that ac-

tion potentials could efficiently be transmitted through the passing direction of the “axon-diodes” [17]. However it is the first time that the blockade of the electrical activity of the neurons in the blocking direction of the “axon-diodes” is formally demonstrated. Our results reinforce the pertinence of the “axon-diodes” in the reconstruction and dynamic study of oriented neuronal networks.

We can notice that the two transfected cultures do not respond in the same way to the stimulations. This can be explained by local differences in the density of neurons or in their clustering state. It can also be due to the fact that cortical cultures at DIV14 presents a spontaneous activity [8]. This spontaneous activity can varies depending on the chips and the local density. This variability make the interpretation of the stimulated activity more complex. The densities and local clustering of the cultures need to be evaluated after live recording and we need to be careful to only compare similar cultures. In addition, using more powerful stimulation (by increasing stimulation duration or LED intensity) may help to reduce this variability.

Our optical method could easily be expanded to more complex neuronal devices, especially in the framework of synaptic plasticity and learning *in vitro*, to determine changes in functional connectivity during development and training.

Localized stimulations and optical fibers

To study the behaviour of complex neuronal devices where several populations have to be stimulated independently, it is necessary to increase the spatial resolution of stimulations.

Although the use of LED arrays, focused on the specimen through the objective of the microscope, allows to evoke activity with high spatial resolution [34, 35], it makes it tributary to the continuous use of a microscope. We rather evaluated the possibility to induce bursting using optical fibers that could be plugged on top of our neuronal cultures.

The experiment was first performed at DIV17 when the spontaneous activity of the cortex was high. Under these conditions it was difficult to sort out evoked from spontaneous bursts. Observing the efficiency of burst induction upon short stimulations was not possible. Instead, we showed that continuous stimulations increased the bursting rate of the culture when compared to the spontaneous activity (n=1 culture, fig. 4.6). This result is not sufficient to conclude about the efficiency of burst induction, but it shows that the luminous power at the output of the fiber is sufficient for stimulations.

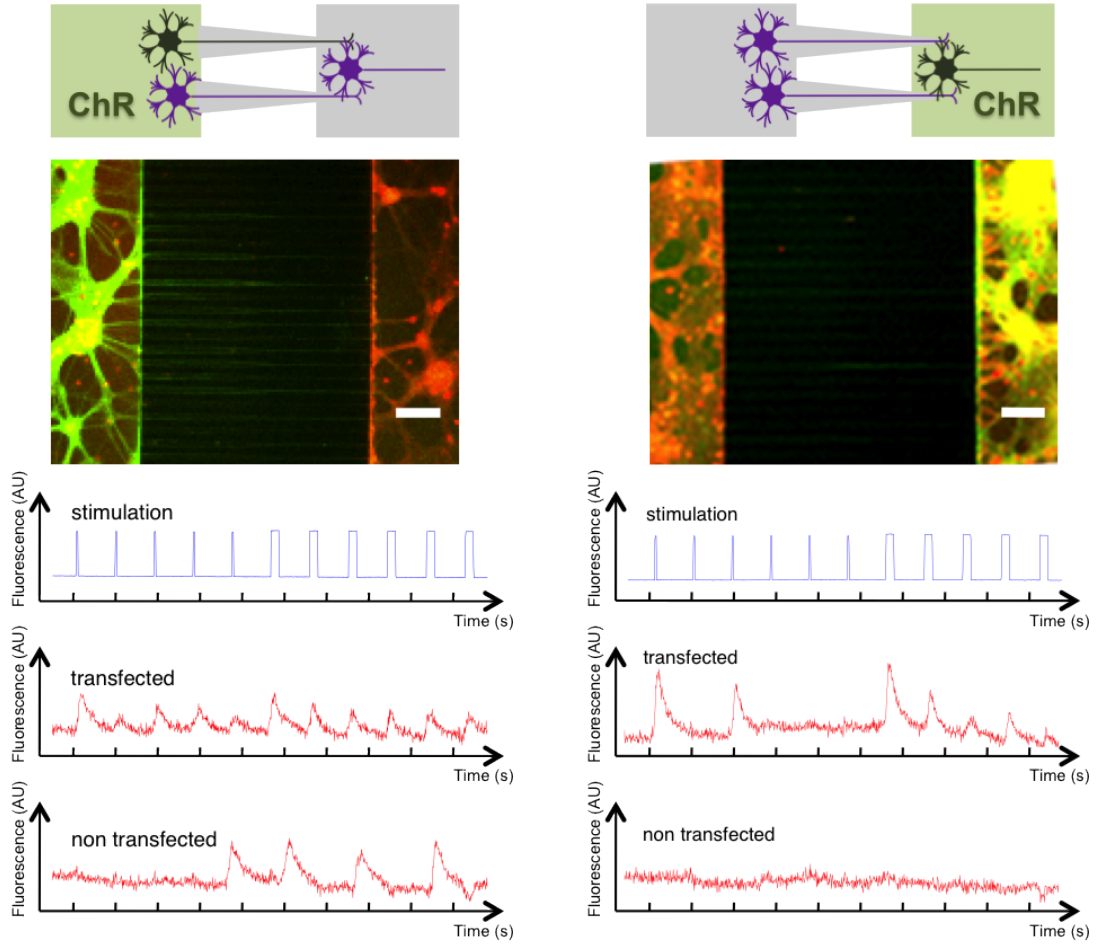


Figure 4.5: Propagation of induced bursting events between two populations connected through “axon-diodes” (DIV14). Pictures: merged fluorescence signal of ChR2-YFP (green channel) and Calcium-Orange (red channel) (scale bar $100\ \mu\text{m}$). Traces (from top to bottom): stimulation pattern, induced activity in a transfected population, spontaneous activity in the non-transfected population but connected to the transfected and stimulated population (stimulations 125 or 625 ms).

Using fibers reduces the luminous power delivered to the neurons and consequently the efficiency of burst induction. However there is still room for improvement on many levels; the LED itself could be more powerful, optical fibers with reduced attenuation could be used, and its coupling to the LED and to the device could be more efficient. In addition, embedding of fibers in neuronal devices would allow reliable control of neuronal activity in complex neuronal devices, whether under the microscope when location of stimulations need to be independent from what is in the field of view (which is not possible when using focused LED arrays), or in an incubator to perform long term stimulation protocols without the need to align or focus the light source on the different population.

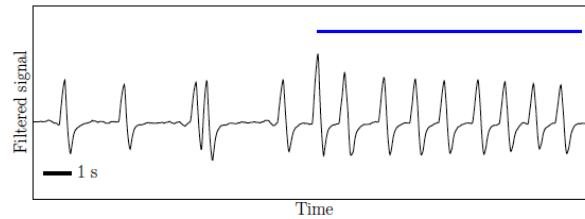


Figure 4.6: Spontaneous activity vs induced activity using optical fiber excitation (DIV17). *The time of stimulation is specified by the continuous blue line above the filtered signal.*

Such embedding of optical fibers in PDMS microfluidic devices was successfully performed.

4.2.5 Conclusion

Calcium-Orange indicator is compatible with the use of cultures transfected with Channelrhodopsin-2. This combination allows simultaneous excitation and recording of neuronal activity. We demonstrated the usefulness of this combination by inferring functional connectivity inside reconstructed neuronal networks. Finally we discussed the use of embedded optical fibers to improve the spatial control of neuronal activity in neuronal devices, independently from the need of a microscope or other optical devices. Our method is low-cost and easy to set up compared to MEA strategies which reinforce its practicality and usefulness for biologists and engineers. This novel fully optical method, used in combination with microfluidic oriented neuronal networks, paves the way to study *in vitro* the relationship between the global activity of networks and cognitive processes.

4.3 Further explorations and perspectives

4.3.1 Towards the living perceptron *in vitro*

Beyond the methodological developments described above, we also tried to build the living perceptron. New chips were created taking advantage of “axon-diodes” and in-mold patterning tools. The design adopted, illustrated in figure 4.7 present five disc shaped isolated seeding compartments for neurons. Three of these isolated sub-populations of neurons, constituting the input layer, can send their axons towards the two others, designed as the output layer. To avoid geometrical biased in the initial synaptic connections of the network, all the pathways connecting input populations to output populations were designed to have exactly the same length.

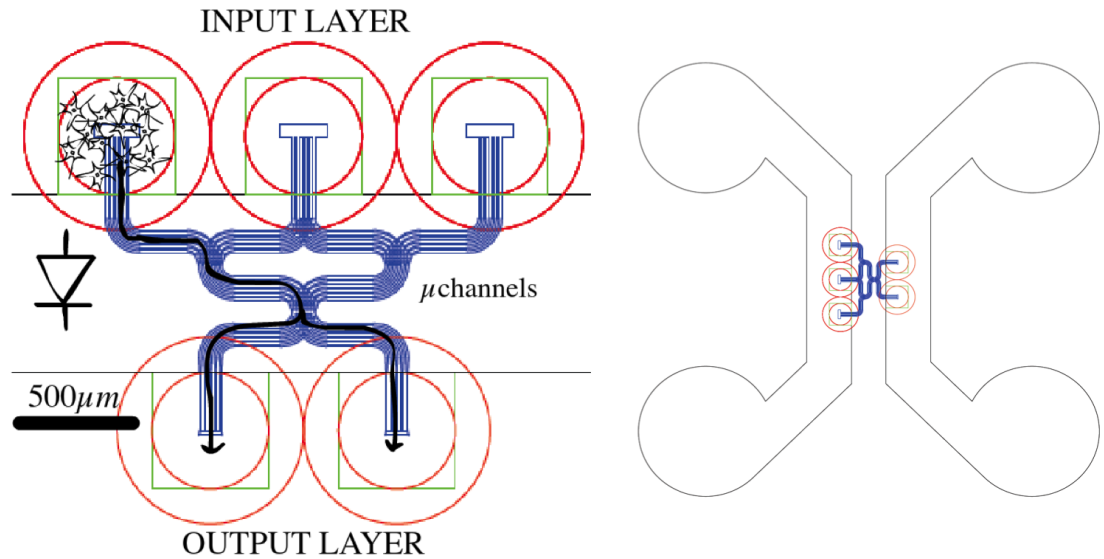


Figure 4.7: QCAD design of the microfluidic chip to build the living perceptron.

The perceptron pattern described above can be coupled with the macro-channels of our regular 2C design. The idea was to create the perceptron pattern and couple it with our usual macro-channels to easily seed and feed the cells. Poly-D-lysine micro-contact printing of the perceptron pattern was first used but the alignment with the macro- and micro-channels was very tedious and not very reproducible so we switched to in-mold patterning. Because the structures created by in-mold patterning are both structured in 3D and coated, it makes it much more easy to align the patterns with microfluidic channels under a binocular. As shown in figure 4.8, this design was successfully seeded with neurons.

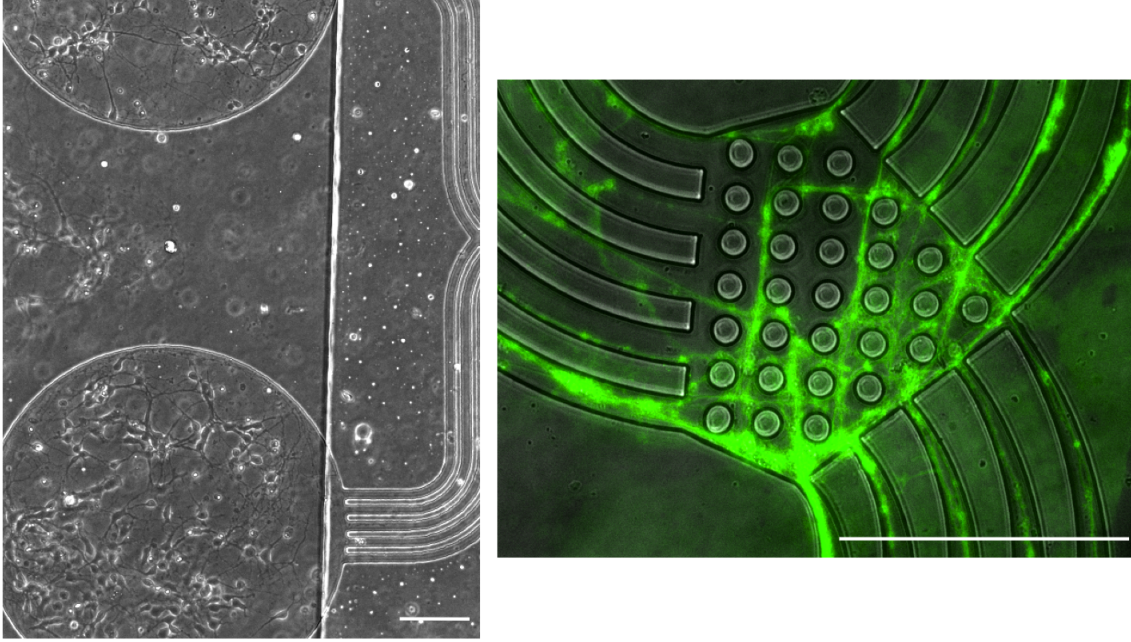


Figure 4.8: Perceptron patterns seeded with cortical neurons DIV6. *Left: translight image (scale bar 100 μm). Right: merged translight image with green fluorescent from ChR2-YFP transfected neurons (scale bar 100 μm).*

“Axon-diodes” are less efficient when implemented by the in-mold patterning technique, but it will not be necessarily crucial in this case. We wanted to use arrays of pillars to shuffle the axons at the joining points between different populations. The pattern we used was not very efficient (fig. 4.8) because axons could cross straight between the pillars. This pattern has to be improved.

Finally the different sub-populations of the network needed to be accessible to optical fibers for the excitation of transfected neurons by blue light. To achieve that a comb with 5 teeth aligned with the perceptron pattern were used to create wells less than 1 mm above the macro-channels ceiling. A scheme representing the final set-up of the chip is presented in figure 4.9. The optical fibers can be introduced in the wells to illuminate one or the other of the neuronal sub-populations (fig. 4.10).

4.3.2 Perspectives on studying dendritic integration

In collaboration with Romain Cazé and Boris Gutkin we brainstormed on another application of our optical stimulation and recording method. This method could be used to study dendritic integration. Dendritic integration is the computation that determines if a given set of input signals from afferent neurons will induce the

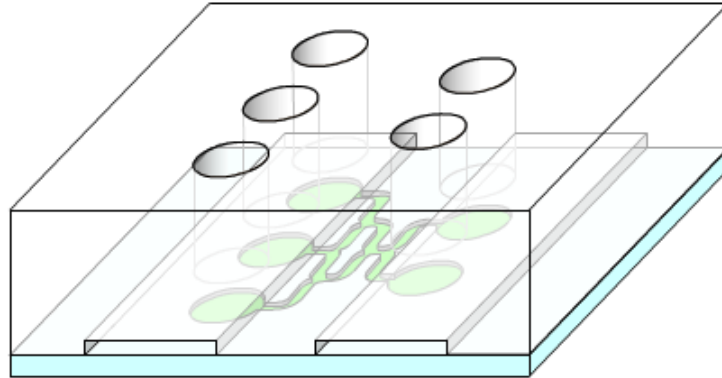


Figure 4.9: 3D representation of the microfluidic chip to build the living perceptron.

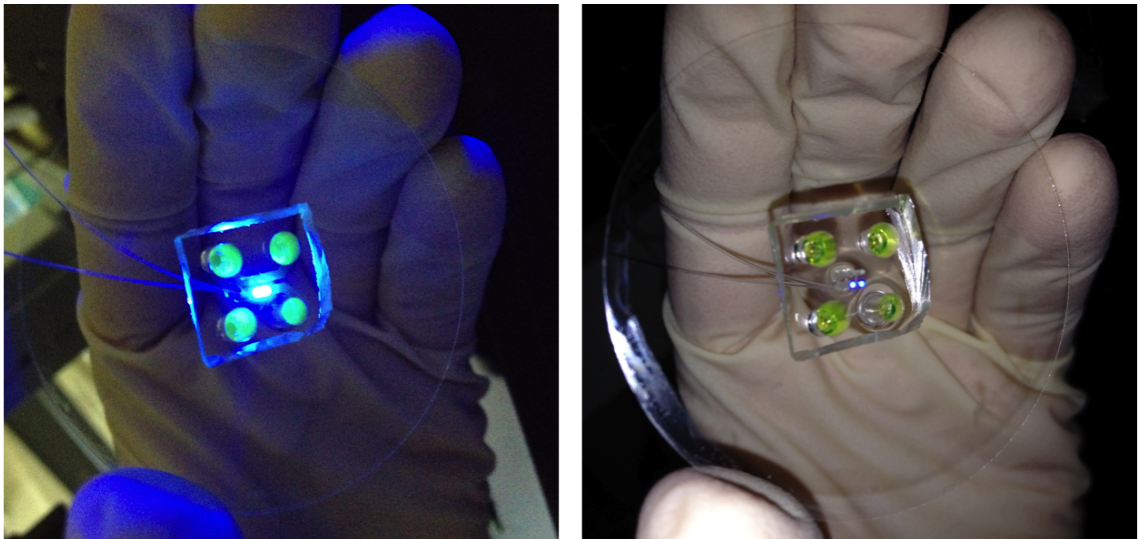


Figure 4.10: Illustration of the blue illumination with two optical fibers embedded in a PDMS chip.

transmission of an output signal to the next neurons [36] (fig. 4.11).

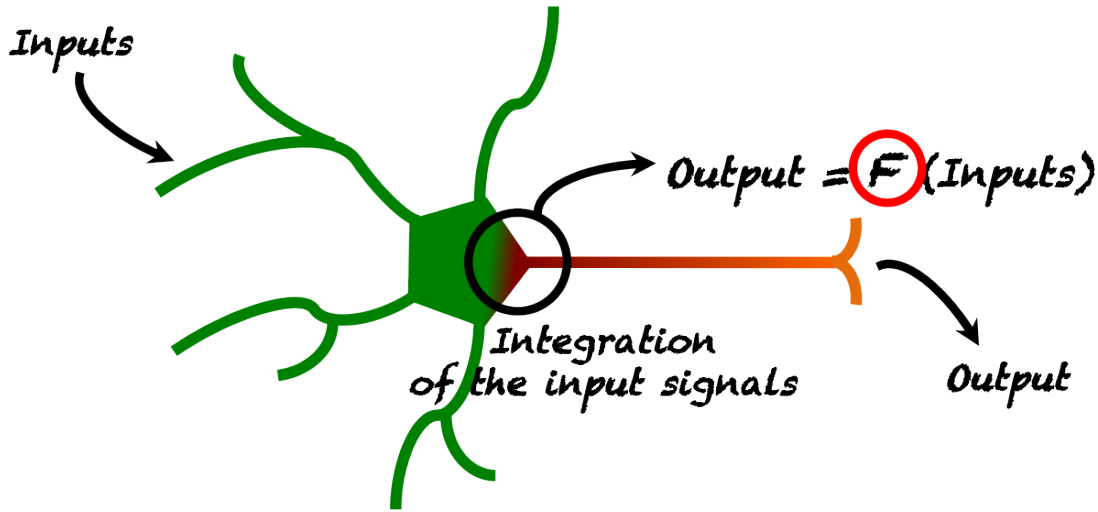


Figure 4.11: Illustration of the concept of dendritic integration.

Romain Cazé studied dendritic integration during his PhD thesis. He built a mathematical and biophysical models to describe the specific cognitive functions that would arise from non-linear dendritic integration (compared to linear dendritic integration) [37].

He demonstrated that even simple neuronal networks can make the difference between linear and non-linear dendritic integration either at the single neuron level or at the level of neuronal populations. The simplest artificial neuronal network allowing to differentiate these two kinds of integrations is composed of two input populations of neurons connected to a single output population. In this model each population should at least show three distinguishable activity modes. If a non-linear integration is implemented in such a network, two novel input/output functions F (fig. 4.11) appear as compared to an assumed linear integration.

Our microfluidic devices could allow us to build such networks comprising two input populations connected to a single output population. Our optical method to stimulate and record neuronal activity would then allow us to enforce three activity patterns on the two input populations. We would then record the activity of the output population and see if we can recognize the non-linear functions. Even if this project remained at the brainstorming stage so far, all the techniques are now available to test the model of Romain in living neuronal networks.

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Part II

Teaching Organic Chemistry through Games

“Il n’y a pas de honte à préférer le bonheur.”
René Char

Chapter 5

Teaching Organic Chemistry through Games

Beforehand, it is important to note that this chapter deals with pedagogy and does not pretend to belong to the science of education. This chapter shows the creation, use and preliminary pedagogical assessment of serious games to teach chemistry. I co-developed these games during my PhD, within the chemistry teaching team of the Frontiers in Life Sciences bachelor of Paris Descartes University.

5.1 Our approach and initial questions

In September 2011, we welcomed our first students in the bachelor program Frontiers in Life Sciences (in french *Frontières du Vivant*, FDV) from Paris Descartes University. This interdisciplinary undergraduate curriculum was founded by a group of young doctors, notably Livio Riboli-Sasco, Claire Ribault and Anne Le Goff, under the impulsion of Francois Taddei at the Center for Interdisciplinary Research (CRI) in Paris. The idea was to train people with an innovative research-based pedagogy. In this very dynamic environment, new pedagogical methods have been experimented ranging from project-based learning to self-learning and peer-teaching.

With Vincent Dahirel, we had the idea of developing and using serious games to teach different core concepts of chemistry. These games are never used alone to approach a notion. They are fully integrated in the curriculum and are associated

with online or classical classroom lecturing, and exercises sessions or practicals. Four games has been developed on atoms, molecules, and organic reactivity. Two inquiry games, respectively on two-dimensional (2D) and three-dimensional (3D) structures of molecules, allow the students to discover by themselves the principles and rules of construction underlying these representations. The two other games, the $\chi\varsigma$ (who's who) on the periodic table and the one on organic reactivity, are helping the students to master some concepts taught in a regular course.

In close collaboration with Livio Riboli-Sasco and Claire Ribault from the "Ateliers des Jours à Venir" cooperative company, as well as actors from game design, research in education (notably David Lafarge [1]), and scientific mediation (notably Richard-Emmanuel Eastes [2]), we begun to evaluate the pedagogical impact of these games in order to make them progressively available to a broader public, going from other undergraduate chemistry programs to general public. The first evaluations described in this chapter are based on a preliminary pre-test/post-test study realized on 36 students of the bachelor program Frontiers in Life Science, who actively participated and greatly helped us to improve our games. The names of all the students cannot be detailed here for concision reasons but can be found in appendixn B page 199.

The chemistry curriculum of the first year of FDV bachelor (L1) is covering basic concepts on atoms and molecules during the first semester, followed by kinetics and organic chemistry during the second semester. The first course of the year is an interactive course alternating frontal parts and exercises on the interactions between the light and atoms and materials. This course is followed by a interactive course that describes the organization of electrons in atoms and the periodic table of elements. During this course the game on the periodic table is used in place of some exercises. To approach the arrangement of electrons in molecules an inquiry game on Lewis is used. The game session is followed by an online interactive self-learning course created by the teaching team. Small exercises are also done in autonomy by the students. The 3D organization of atoms in molecules is then introduced using another inquiry game. Again the game session is followed by a self-learning session with a course and small exercises. After these courses a frontal course is given on molecular orbitals and that ends the first semester.

During the second semester, our students follow in parallel a course on kinetics and a course on organic chemistry. The two courses are usually taught by two different teachers but bridges are built between them and they enrich each other nicely. A first introductory frontal course on the basic reactivity of molecules, including the fact that some molecules are sources of electrons (nucleophiles) or sinks of electrons (electrophiles) is given first. Then instead of doing a normal

exercises session, we use the game on the reactivity of molecules to help the students understanding these abstract and difficult notions.

5.2 Description of the games

In this section, I will present the rules and pedagogical aim of each game. A summary of the main characteristics of our games is shown in the table of figure .

5.2.1 The periodic table $\chi\varsigma$ (who's who) game

Overview

Pedagogical aim: learning the properties of the elements according to their position in the periodic table.

The rules of this game are copy-pasted from the world spread game “Who’s Who?” (“Qui est-ce?” in french, fig. 5.1). In this game you have a set of drawn portraits in front of you, including one that has been secretly picked by your contender. While asking yes or no questions about the physical traits of the different drawn characters, you need to guess the portrait he or she picked. In our game the elements of the periodic table replace the drawn portraits and players have to ask questions about the chemical properties of these elements.



Figure 5.1: Picture of the box of the french version of the game “Qui Est-ce?”. (Credits: <http://www.eighnies.fr/jeux-et-jouets/348-qui-est-ce>).

Rules

Goal

Guessing the chemical element of the periodic table that has been picked by the other player.

Materials

- Two big (A2-sized) boards representing the periodic table of the elements (fig. 5.4).
- Three sets of cards representing all the chemical elements of the periodic table with size that fits the cells of the big periodic tables. These cards contain the abbreviated name of the elements as well as some of their main chemical characteristics such as their atomic number, mass number, electronegativity value, ...etc... (fig. 5.2 and fig. 5.3).

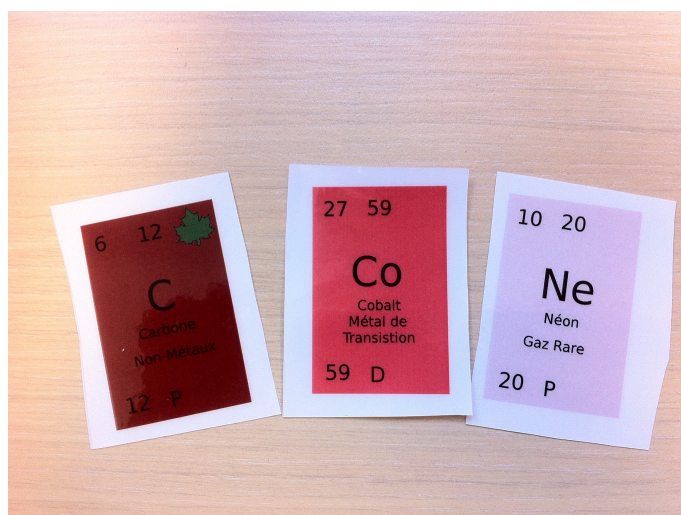
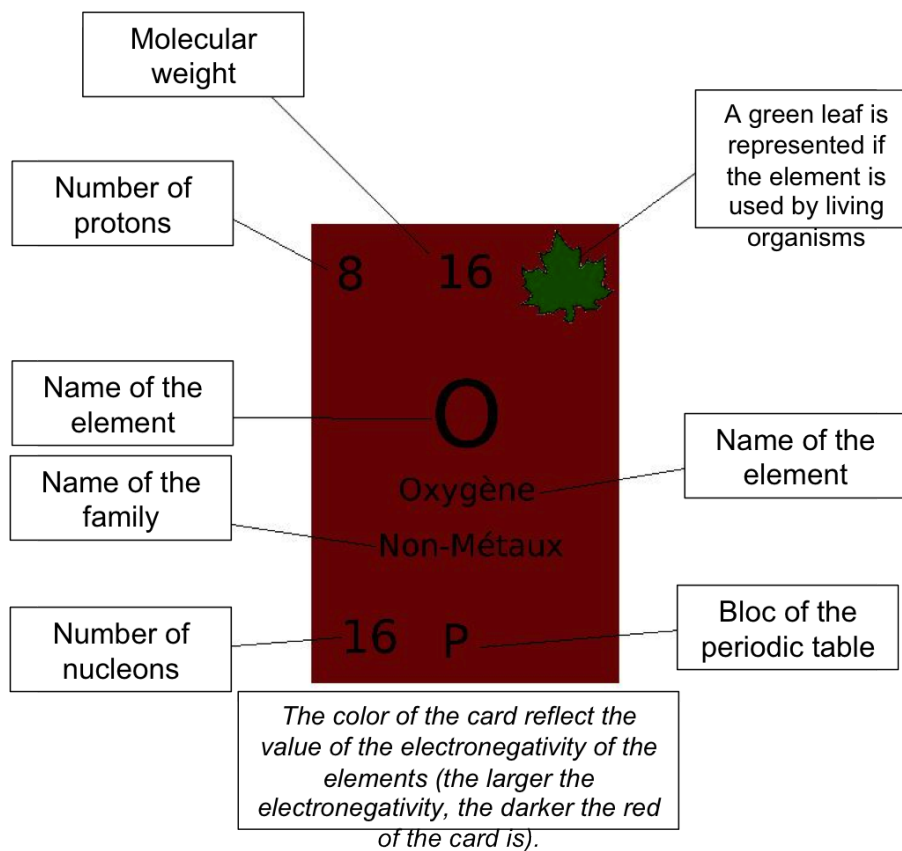


Figure 5.2: Example of cards of the χ_S game.

Setting Up

This game is designed for two players.

At the beginning of the game each player receives a periodic table board and a set of cards representing the chemical elements that he or she needs to organize on top of the periodic table board (fig. 5.4).

Figure 5.3: Description of the card layout in the χ_5 game.Figure 5.4: Picture of the set up of the χ_5 game.

Using the third set of cards representing the chemical elements, each player needs to secretly pick an element of his choice and keep it hidden from the other player.

Structure of play

- Players alternate turns.
- During a player's turn, he or she asks a yes or no question about the chemical elements secretly picked by his or her opponent. According to the answer to that question, the player flip the card(s) of the element(s) of the periodic table that does(do) not present the corresponding revealed chemical characteristic.

Winning

The first player to guess the element secretly picked by his or her opponent wins the game.

5.2.2 The games on the 2D and 3D representations of molecules

Brief presentation of the 2D and 3D representations of molecules

Molecules are groups of atoms that assemble by covalent bonds and constitute stable chemical entities. Molecules are microscopic elements with sizes ranging from hundreds of picometers for simple molecules with two atoms, to several nanometers for the biggest proteins. To visualize and mentally manipulate molecules it is necessary to build comprehensible representations.

Molecules are classically represented in 2D or 3D as illustrated in the figure 5.5. The 2D representations of molecules, showing atom symbols, the covalent bonds between them and lone pairs of electrons, are commonly named Lewis representations, after the chemist who developed the rules underlying them. The core rule used to build Lewis representations is to organize the electrons shared by the atoms of the molecule (called valence electrons) so that the maximum of atoms are surrounded by 8 electrons. This rule is called the “octet” rule and have some exceptions that I will not discuss here.

The 3D representations, giving an idea of the organization of the molecules in space (with lines representing bonds in one main plane, plain triangles pointing to atoms located in front of the main plane and dotted triangles pointing to atoms located behind the main plane), are called Cram representations. The theory used to build these representations is named the Valence Shell Electronic Pair Repulsion (VSEPR) theory. The VSEPR theory stipulates that given a molecule (or part of

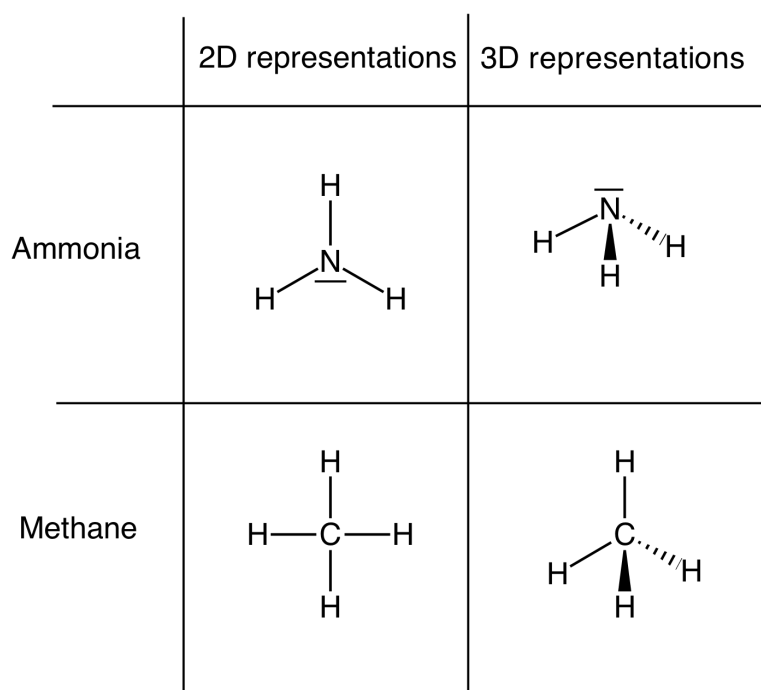


Figure 5.5: 2D and 3D molecular representations of ammonia and methane. *Lines represent covalent bonds or lone pairs (on the nitrogen atom) that are in the plane of the sheet. Plain triangles are pointing to atoms located in front of the sheet plane and dotted triangles are pointing to atoms located behind the sheet plane.*

a molecule) with a central atom the electron pairs surrounding this atom (covalent bonds or lone pairs) are repulsing each other and try to maximise the distances between them.

In the following sections, the games developed on the 2D and 3D representations of molecules are respectively called the Lewis and VSEPR games.

It is important to notice that the rules underlying Lewis and Cram representations are not very complicated and can thus easily be guessed by undergraduate students. More complex systems with more complicated rules would maybe not be appropriate to approach through the same kind of inquiry games.

Lewis Game Overview

Pedagogical aim: learning the concepts and rules used to build the 2D representations of molecules and the repartition of electrons between atoms in a molecule.

The Lewis game is an inquiry game during which students are lead to discover by themselves the rules underlying Lewis representations simply by observing cards showing Lewis representations of simple molecules. The students are organized in teams of around 4 players and the team who wins is the one who guess the most rules and define the most precisely. This game is adapted to group of students with an heterogeneous level in chemistry and takes into account that some students with original curriculum might already know Lewis representations. In its format, this game also recapitulates the different steps of the experimental research method.

Lewis Game Rules

Materials

- One set of cards showing Lewis representations of diverse simple molecules (fig. 5.6).
- Counters or “coins” (beans, chips, paper coins...).
- Two spaces phonically isolated from each other (typically two separated rooms).
- Paper and pens.

Assigning roles and teams

The game is designed for 10 to 30 students. Students are divided in two groups: (1) the students who don't know Lewis representations yet that will play as “novices”

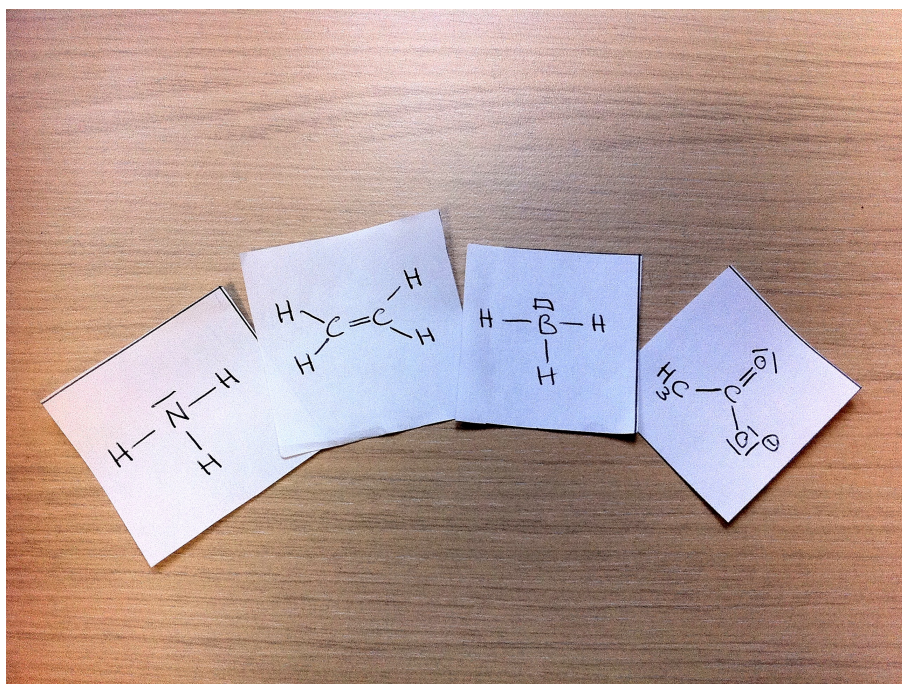


Figure 5.6: Examples of cards of the Lewis game.

in the game, and (2) the students who know already Lewis representation that will play as “wise men or women” in the game.

The wise men and women constitute a team. The other players are organized in teams of 3 to 5 players. The teams can be randomly formed, formed by affinity (which was the case in our classrooms) or formed by the teacher.

Goal

Novices goal Guessing the rules underlying Lewis representations of molecules.

Wise men and women goal Helping the novices teams so that they guess the maximum of rules and enunciate them well.

Setting Up

At the beginning of the game, each team of novices receive three cards showing the 2D representations of three different molecules. The molecules are not choosen randomly, they need to contain at least one lone pair, one double bond and if possible one “lacune électronique or one formal charge.

Each team also receive 8 coins that will be used as “money” to pay advices from the wise men and women.

Structure of play

The game is divided in three phases:

First phase The novices begin to guess the rules without any guidelines. In a separated room, the wise men and women discuss and agree on the rules that the novices need to guess. This phase last approximately 15 minutes.

Second phase The wise men and women join the room where the novices play. Novices team can now ask for advices (as described below) from the wise men and women as long as they have enough money to pay for them. This phase last approximately 20 minutes.

Third and scoring phase The rules enunciated by the different novices teams are collected and the scores of the different teams are determined with the whole class. This phase last approximately 20 minutes.

The wise men and women can give four different kind of advices to the novices teams:

Additional card The novices can ask for an additional card that can be picked by the wise men or women. They can also ask for a card with a specific characteristic if they wish (double bond, lone pairs, charges...). Any additional card costs one coin.

Yes or No Question The novices can ask a yes or no question for 2 coins.

True or False Rule The novices can formulate a rule that they have guessed to a wise men or women who will tell them if this rule is true or false. Any action of this kind costs 2 coins.

Wise advice The novices can ask for an wise advice that will be specially tailored to boost their progression. This constitutes a big help and it costs 4 coins.

Scoring

During the third and last phase of the game, the rules guessed by the different teams are gathered and presented to everyone. The true rules are refine or reformulate if necessary. The number of points is determined by the teacher and the students according to the degree of importance of the rule. To get the maximum of points for a given rule, a team needs to have enunciate it completely and precisely, otherwise they get only a fraction of the total points. False rules are not penalized

and remaining coins are a priori not taken into accounts in the scoring of the teams.

Winning

The team with the most points win the game but it is important to stress that winning is not really the point of the game ! The teacher should also stress the quality of each team and raise what could have been beneficial for some teams to score better in the game.

VSEPR Game Overview

Pedagogical aim: learning the concepts and rules used to represent the 3D organization of molecules.

VSEPR Game Rules

The VSEPR game is built on the exactly same model as the Lewis game. The only difference is that the cards show molecules in their Cram (3D) representations (fig 5.7).



Figure 5.7: Examples of cards of the VSEPR game.

5.2.3 The electrophile/nucleophile game

Brief presentation of the basic reactivity of molecules

When different molecules are mixed in a pot, it is important to predict who is going to react with whom first to have an idea of the products formed by the reactions. Thus we need to find means to compare the reactivity of different molecules, or even different groups of atoms in a molecule.

The chemical reaction is a transfer of electrons between two molecules. Thus there are two kinds of reactants: (1) reactants that can easily give electrons during reactions, which are called nucleophile (literally “who like the nuclei”), and (2) molecules that attract electrons during reactions, which are called electrophile (literally “who like electrons”).

It is thus important for students to learn to recognize electrophiles and nucleophiles and moreover to appreciate the relative electrophile/nucleophile power of diverse molecules of the same families. Some qualitative concepts can help the chemists. For example, nucleophiles are better if they contains more electrons (lone pairs), if they are negatively charged or if they are bigger. On the contrary, electrophiles are better if they are poor in electrons, positively charged or smaller. However, these “rules” are only qualitative and it is important, but yet usually difficult, to develop an empirical intuition about these reactivity notions. Usually, students build their intuition while doing ranking exercises and practical sessions. We present here a game that can replace or complement exercises sessions.

Overview of the reactivity game

Pedagogical aim: learning the basic concepts underlying the different reactivity of species in organic chemistry.

The electrophile/nucleophile is a card game and is also called by our students “Nucleo-Belote” in reference to the french card game “Belote” that is similar to “Tarot”. The cards of this game imitates a classical card deck where the black and red families are replaced by two families of nucleophiles and two families of electrophiles (fig. 5.8).

The students are invited to transpose and eventually adapt the rules of classical card games to this special set of cards. Our students adapted the rules of several simple card games such as “Bataille” (“War” in english), “Belote” (similar to “Tarot” or “Hearts”) or “President” (similar to “Tichu”). In the following section I will present a simple adaptation of the “Belote” game but keep in mind that many other rules can be adapted or designed to play with our cards.

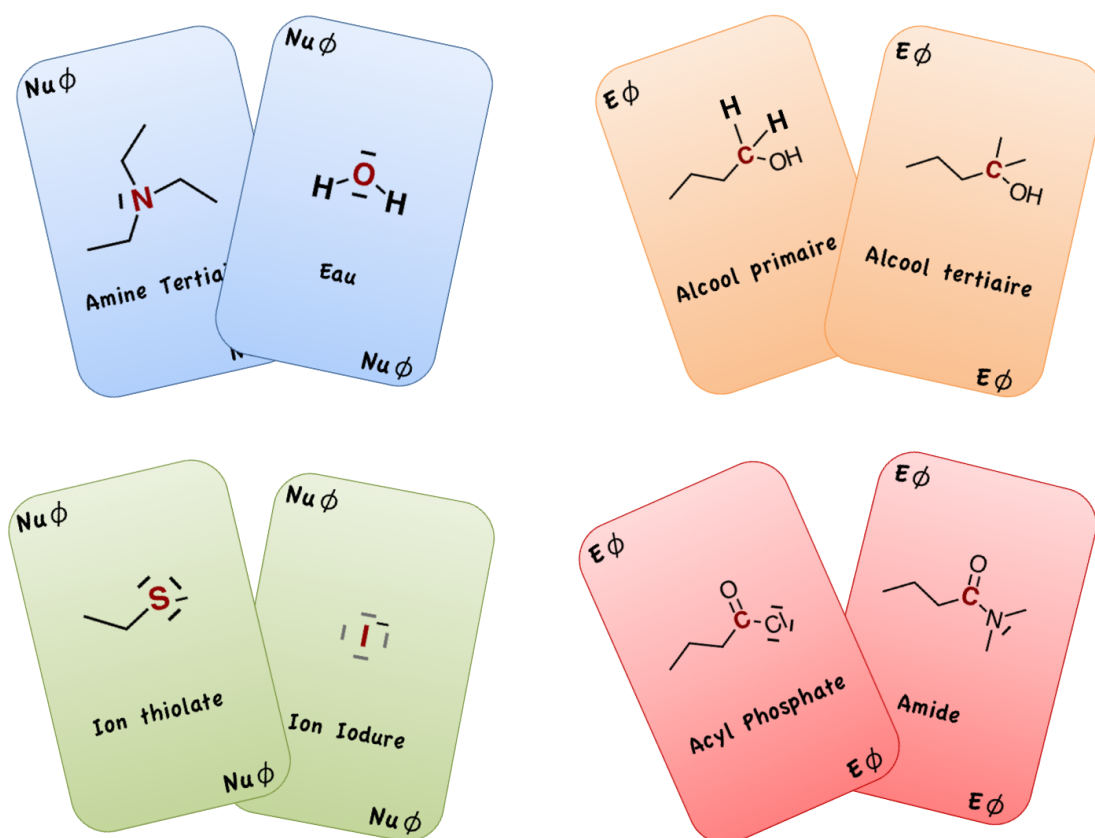


Figure 5.8: Examples of cards of the Nucleo-Belote game.

Example of Rules: the “Nucleo-Belote” rules***Goal***

Winning the maximum of tricks.

Materials

- One set of cards showing families of electrophile and nucleophile molecules (fig. 5.8).

Setting Up

This game is designed for 4 players playing individually or in teams of 2 players.

At the beginning of the game, the cards are evenly distributed among the four players.

Structure of play

The game is organized in rounds or tricks. During a round a first player choose a card and put it face up on the table. The other players play in turn. They need to put a card of the same family of the first one if they can.

The player who plays the most electrophile or the most nucleophile molecules of the given family, wins the trick. If some players putted a card that is not of the same family, they have no chance to win the trick.

The player who wins the trick starts the next round and thus choose the first card of the next round.

Winning

The player who won the most tricks win the game.

Now that all the games are presented, we can move on to the preliminary evaluations that we realized to assess the pedagogical impact of our games.

5.3 Preliminary evaluation of the games

The games were created progressively. The VSEPR, χ_S and Nucelo-Belote games were created for the first promotion of students in 2011. The Lewis game was only

used the year after. The first year, qualitative data only was collected, gathering the feelings of the students about the games.

The appreciations about the games were heterogeneous among students. Some had a lot of fun, some didn't like the games and some had the feeling we were treating them as kids while asking them to play a game. The answers to the question if the games helped them to learn were also heterogeneous. However, the results of the students in the following exams, on the concepts learned by the games were very good. That encourages us to continue to use the game and to start to evaluate their pedagogical impacts.

5.3.1 Methodology

The results presented here were collected by pre-game and post-game surveys on the second generation (promotion 2012) of students of the FDV bachelor. Some data were also collected from the first generation of students to have an idea of the long-term memorization of the knowledge they acquired by the games. The third generation of students already started playing some games and the same surveys has been used to collect more data. However these freshly collected data has not yet been analysed.

Two kinds of evaluation was made:

1. A general evaluation of the appreciation of the games by the students. We mainly wanted to know: how much they liked the games, how much they had fun and how they feel the games helped them to learn.
2. An evaluation of the pedagogical impact of each game. We wanted to test the knowledge and competences, as well as the memorization of the concepts learned by the games.

In FDV bachelor, each promotion count 18 students. This small number limits the significance of our results but it was good enough for a preliminary study. Due to this small number of students we decided not to have controlled groups. This decision limits the significance of the results. Starting in september 2014, the number of students per promotion is going to increase to 30. It will then be possible to divide the classroom in two groups and have a control group. The possibility to try to use our games in other curriculum with more students is also envisioned.

The surveys were implemented on the online teaching platform called "Learning Catalytics" [3] (fig. 5.9), developed by Martin Vogt and Eric Mazur Professor of Physics at Harvard University, and recently acquired by Pearson. This platform is

very convenient for various reasons. (1) The variety of type of questions that can be asked is huge, ranging from checklists to drawings (fig. 5.10). (2) Questions can be shared with the community of teachers using “Learning Catalytics”. This way, if other teachers use one of the questions you developed (or vice-versa) you can have an idea of how your students perform compare to other groups of students. (3) Students have a personal account and after they answer the survey you can retrieve and download all the answers per students or per questions very easily. (4) The platform is user friendly, insure the confidentiality of the data and offered so far good support services.

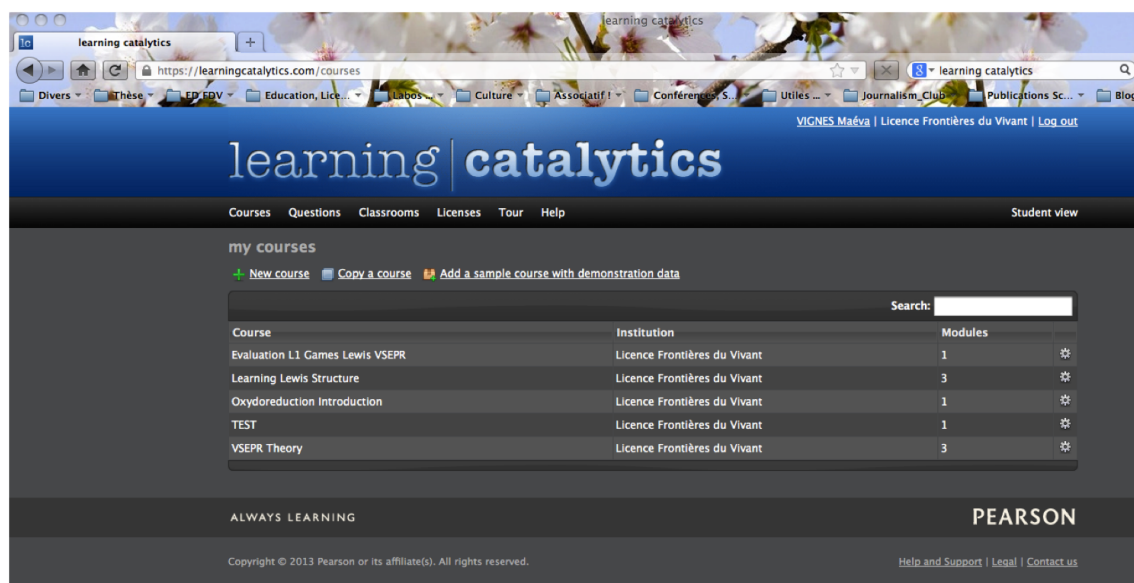


Figure 5.9: Screenshot of my home page on the website Learning Catalytics (<https://learningcatalytics.com>).

All the surveys used in this study can be found in the appendix C page 201. The surveys contain two main types of questions: (i) questions about students appreciation of the games and (ii) questions to test the knowledge of the students on the chemical concepts introduced in the game. The surveys also contain questions about chemical concepts that are not directly linked to the game and has been taught using more classical pedagogy. There are also questions to test the general scientific background of the students. These two last type of questions constitute “control questions” respectively inside the chemistry discipline and outside of the chemistry discipline. The questions of each survey were divided into relevant categories that are presented in each subsections presenting the results on one particular game.

All statistical analysis were conducted using program R [4]. For the questions testing the evolution of the knowledge of the students along their learning path, lin-

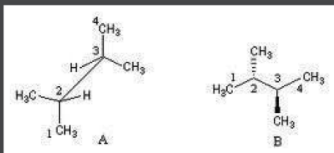
1. multiple choice

Have you already heard about VSEPR representations or theory?

- Yes and I master it
- Yes I am familiar with it
- Yes a little bit
- Not at all

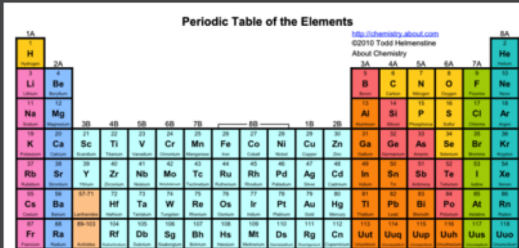
2. region

Click on the VSEPR representation of the molecule.



3. region

Click on the block s of this periodic table.

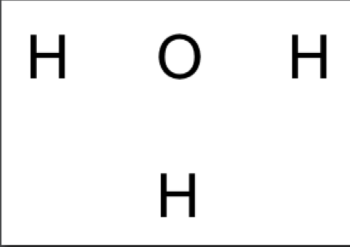


11. short answer

What is the most fundamental rule of VSEPR theory?

12. sketch

Draw the Lewis structure of the H_3O^+ ion. Don't forget lone pairs and charges.



13. multiple choice

What is required in the axon terminal to start neurotransmitter release?

- Increased intracellular Na generated during an AP
- Decreased intracellular K generated during an AP
- An increase in membrane potential generated during an AP
- All of the above

Figure 5.10: Examples of questions created on the Learning Catalytics website (<https://learningcatalytics.com>).

ear models (lm function in R) were used to assess whether the differences observed were significant or not. For these questions the means over all students and all questions of one given category were plotted, as well as the standard error of the means over the different questions of one given category.

The data collected on the Nucleo-Belote game was a special case. This data was kindly collected by a collaborator in Columbia, Prof. Ana Maria Campos from the University Jorge Tadeo Lozano of Bogotá. The survey was designed by me and realized using “Google Survey” by Ana Maria Campos on 24 students of the organic chemistry course of the first year of a chemical and biological engineering curriculum.

It is important to note that for the moment, the data collected were only analysed per questions. The analysis per students has not been done yet but will be done in the future.

5.3.2 General appreciation of the games

In this section, we present how our students perceived the learning through games pedagogy. We tried to evaluate how much they liked the games (also in comparison with other pedagogical approaches), and how much they felt they learned through the games.

The games are globally appreciated by the students. Among the 2012 promotions, around 70% of the students said that they liked the games much or very much. When they were asked which game they liked the most, 50% of them answered the Nucleo-Belote game. The Lewis and VSEPR games were appreciated the same way by the students, 20% of students chose each of them as their favourite game. Finally, the $\chi\varsigma$ was the least preferred of our games.

Given the variety of type of courses provided to students in the FDV bachelor, we wanted to evaluate how much the students appreciated the games, as compared to other type of pedagogy. Among the 18 students of the 2012 promotion, the learning through games pedagogical approach was their second favourite one at the end of the first semester and their third favourite one after the second semester. After the end of the second semester, the learning through games approach was third after the learning through projects approach and the learning through internships. It is important to notice that our students start to go for internships in labs during the second semester of their first year (two internships of one week).

We also wanted to evaluate how much the students felt that the games helped them to learn and understand the notions introduced. Their feedback was again very positive with only 15% of students saying that the games did not helped them to learn and understand the notions introduced. It is interesting to notice that the perception of the students about how much the games helped them to learn

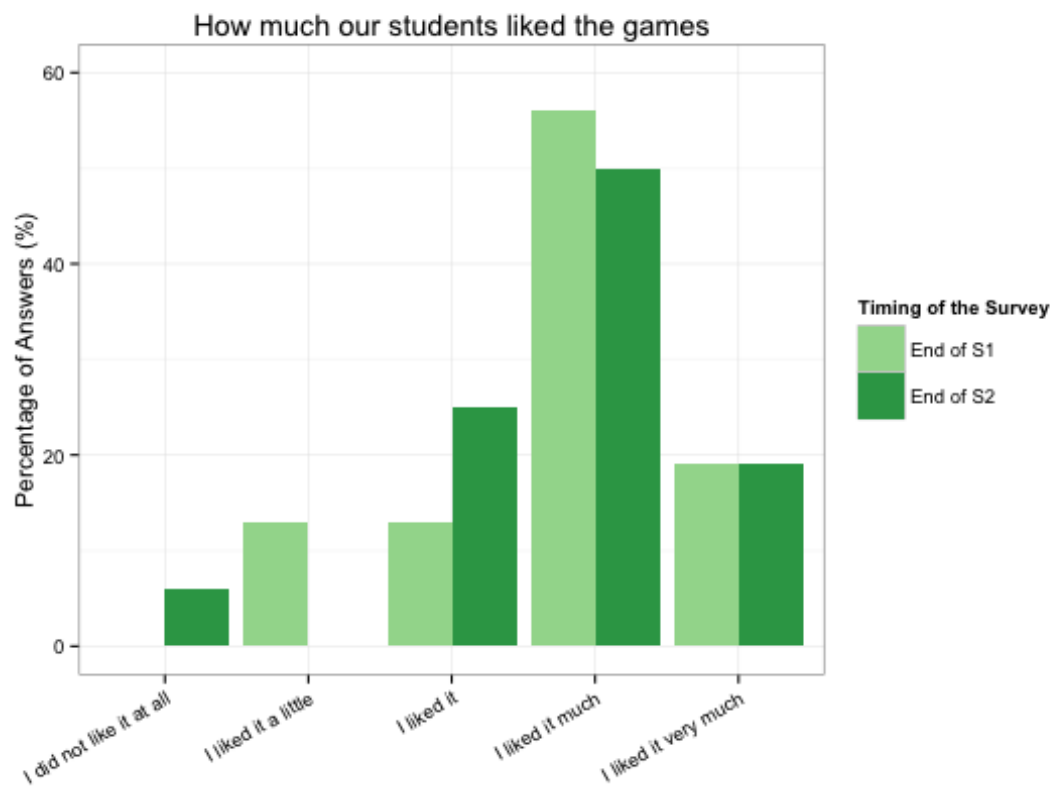


Figure 5.11: Illustration of how much our students liked the games (n=18).

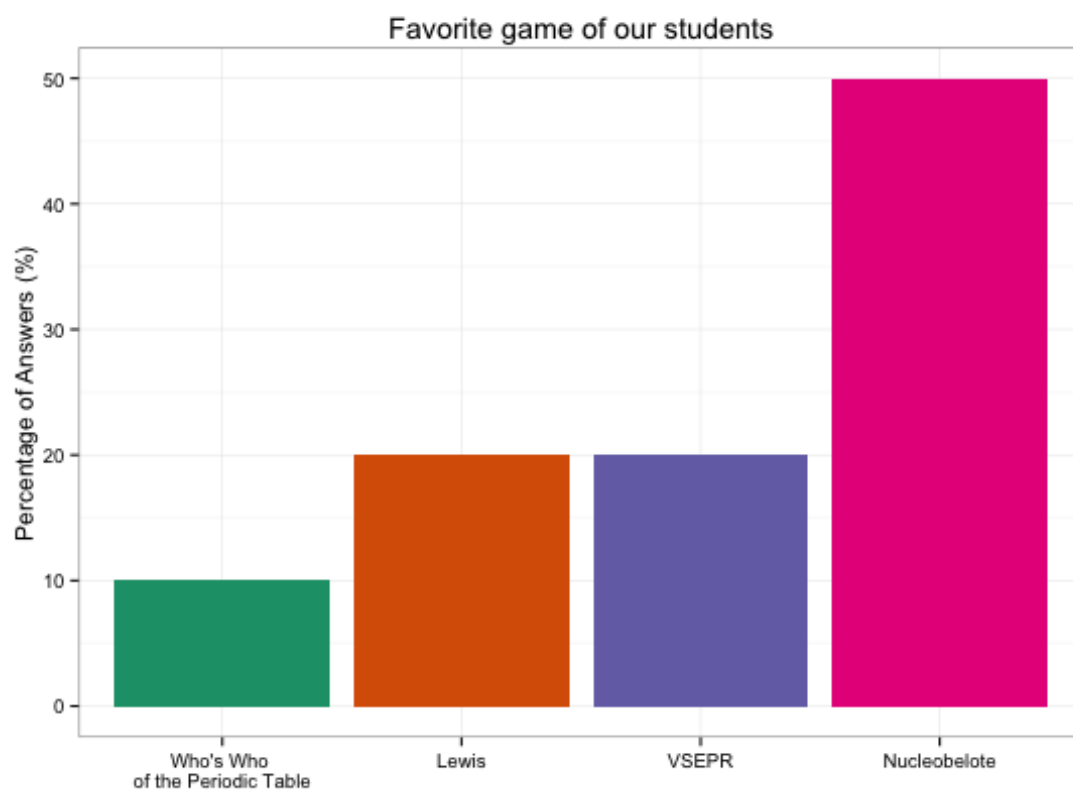


Figure 5.12: The average favorite game of our students (n=36).

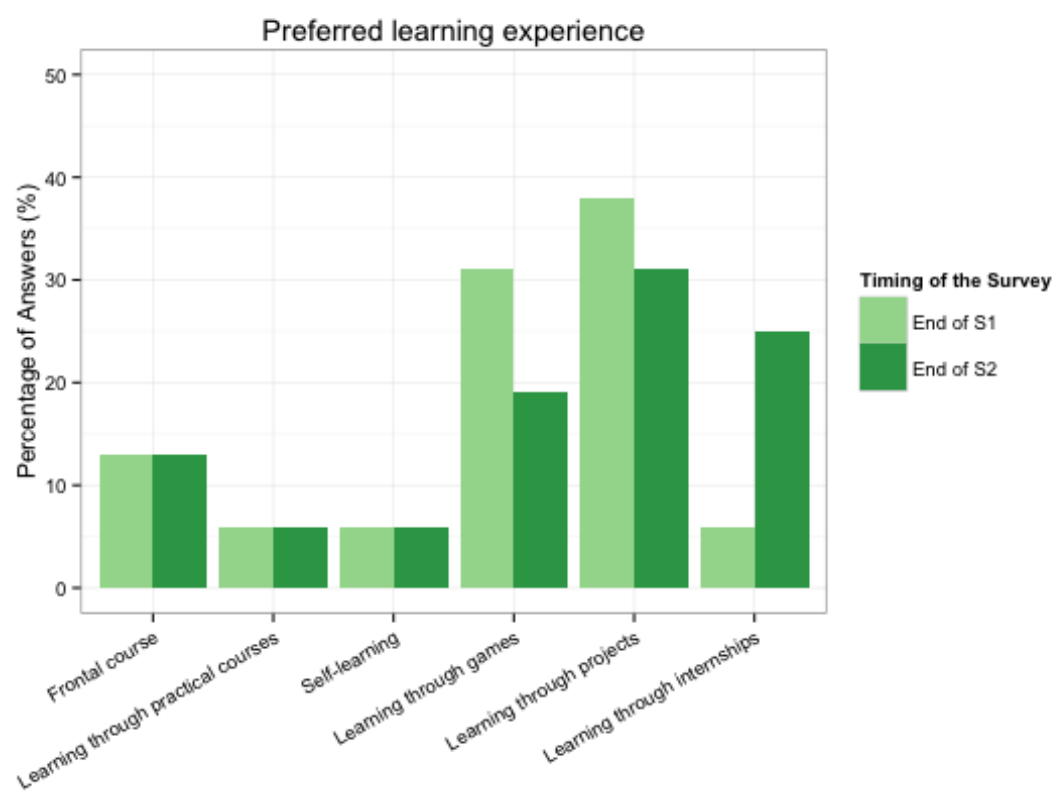


Figure 5.13: Illustration of what types of pedagogy our students like the most (n=18).

or understand is evolving a bit from one semester to the next one. Students can appreciate differently their knowledge while reusing it in other contexts. After some time they might evaluate better how much the games helped or not as compared to other courses or exercises.

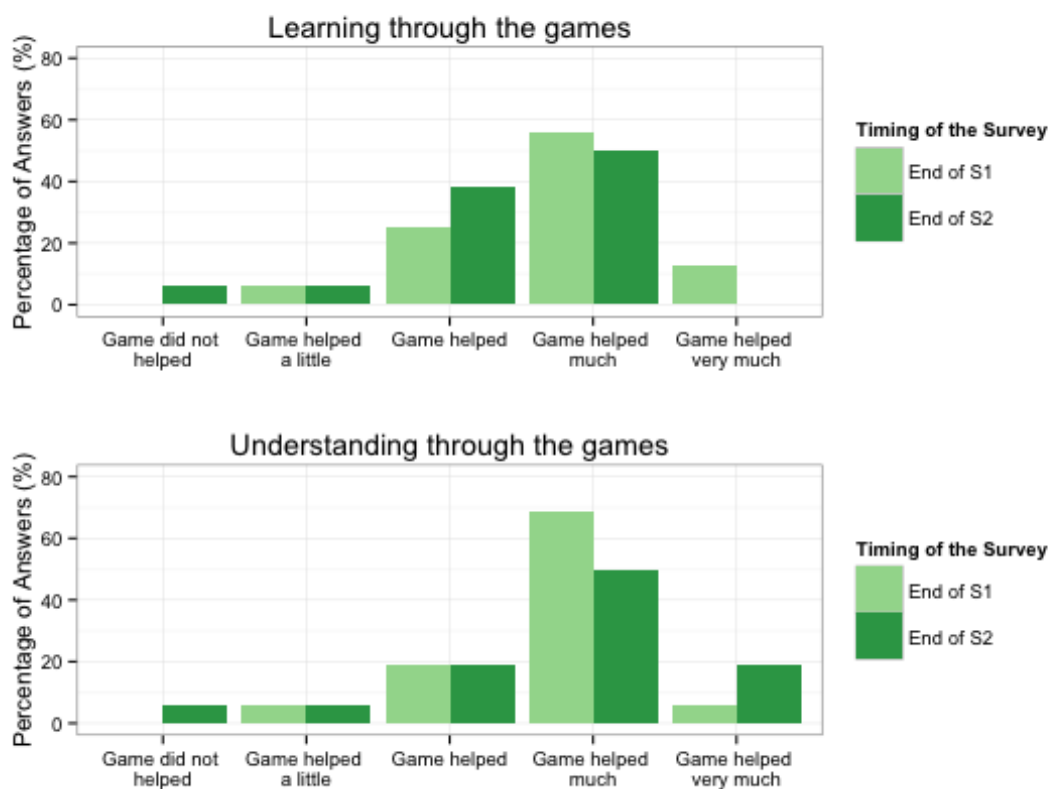


Figure 5.14: Illustration of how much our students felt that the games helped them to understand versus learn new concepts (n=18).

5.3.3 Zoom on Lewis and VSEPR Game

Evaluation of the Lewis game

The survey to evaluate the Lewis game contained 21 questions with 5 questions about the perceptions of the students and 16 questions testing the knowledge of the students on various topics. These topics were divided in 5 categories:

- Lewis topic with 6 questions
- Diverse topics testing the general scientific background (GSB) of the students (excluding chemistry) with 4 questions

- Basic chemistry topic (periodic table, atomic orbitals...) with 3 questions
- A topic on general molecular representations with 2 questions
- Finally 1 question on VSEPR theory

The questions on Lewis and molecular representations allowed us to test the evolution of the knowledge and competences of the students about notions tackled in the games. The questions on basic chemistry and general scientific background were used as control questions.

Students were asked to answer the survey (with always exactly the same questions) just before playing the Lewis game, right after playing the game and finally after having completed in autonomy an online courses and some simple exercises on Lewis representations. The Lewis game was the first “course” on Lewis representations.

The evolution of the average score of the 16 students surveyed along this timing are presented in figure 5.15. The averages are calculated among the 16 students and pulled per topics. Thus, the error bars, that represent the standard deviation of the means, are calculated over the different questions belonging to the same topic.

We can see that there is a tendency of gradual increase of the scores of the students for the two topics that are related to the Lewis game (Lewis topic and molecular representation) after the game and even more after the course. In comparison, the scores in the two unrelated topics of basic chemistry and GSB that seem to remain equals despite the fact that the questions remained exactly the same. However, none of the increase is significant. This might be explained by the fact that the level of the questions was too low or by the too small sample size. We can notice that on average, even before playing the game, around 60% of the answers are correct on the questions about Lewis. Some students in the promotion (around one fourth of the students) were coming from curriculum where they already learned Lewis rules. It might be worth trying to increase the difficulty of some questions and analyse the progression of students depending on their curriculum.

We can notice as well that there is an increase of correct answer on the unique question on VSEPR theory, which is a notion that will be introduced later in the year with the VSEPR game. It might be due to the fact that the students started discussing about 3D organization of molecules while thinking about the rules underlying 2D representations of molecules. It could also be due to the fact that the questions were exactly the same before and after the games, allowing the students to inform themselves on the questions and found out the right answers.

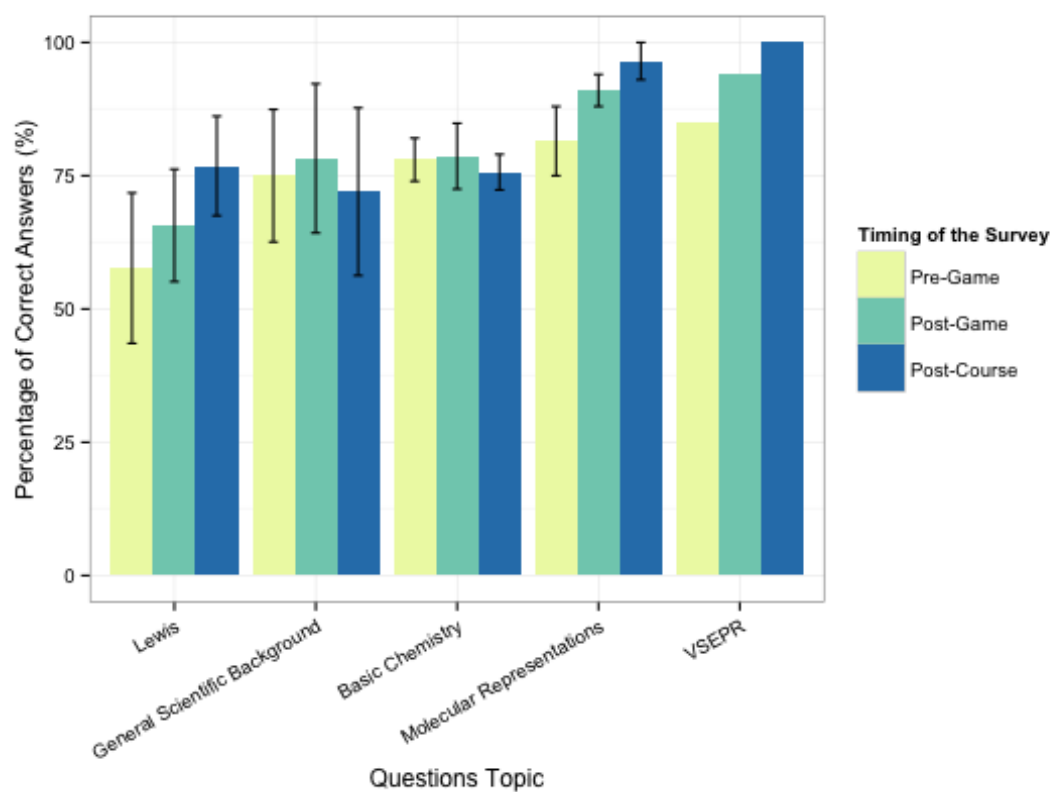


Figure 5.15: Illustration of the evolution of the knowledge of our students before and after playing the Lewis game (n=16).

If the evolution of the scores are plotted separately for each question on Lewis (data not shown), some interesting variability emerge. For some questions, the scores of the students increase after the game and decrease again after the course (while not reaching the pre-game level again). For some other questions, the reverse phenomenon is happening. It seems that some particular notions are better grasped by the students through the game than through the course and vice-versa. Even though the number of students is low and these variations might just be due to noise, it is interesting to keep these data in mind to improve our game and course.

This preliminary study on the Lewis game was mainly interesting in two ways. First, it suggests a positive pedagogical impacts of the game, even though this result will need to be refine with future studies. Second, this first pre-test post-test approach gives us ways to improve our analysis method. It made us realize the importance of the level of difficulty of the questions asked and the importance of an analysis per students. The fact that the questions asked before and after the game were exactly the same was also a limitation that we corrected in the next survey that we built to evaluate the impact of the VSEPR game.

Evaluation of the VSEPR game

The survey to evaluate the VSEPR game contained 20 questions with 2 questions on the impressions of the students and 18 questions testing the knowledge of the students on various topics. The topics were divided in 4 categories:

- VSEPR topic with 9 questions
- Diverse topics testing the general scientific background (GSB) of the students (excluding chemistry) with 4 questions
- Lewis topic with 2 questions
- Basic chemistry topic (periodic table, atomic orbitals...) with 3 questions

18 students were surveyed according to the same timing as for the Lewis game. After discussion with social scientists, we were advised to slightly change the questions in the post-game survey as compared to the pre-game survey. For example, when students where asked to draw the Cram representation of a molecule, the molecule of the pre-game survey was different from the one of the post-game survey (O_2 and CO_2). For questions about notions, the text of the questions were slightly changed. The order of the questions was also different in the two surveys.

The evolution of the average score of the students before the game, after the game and after the course learned in autonomy, are presented in figure 5.16. As for Lewis, the averages are calculated among the 18 students and pulled per topics.

The error bars are calculated over the different questions belonging to the same topic.

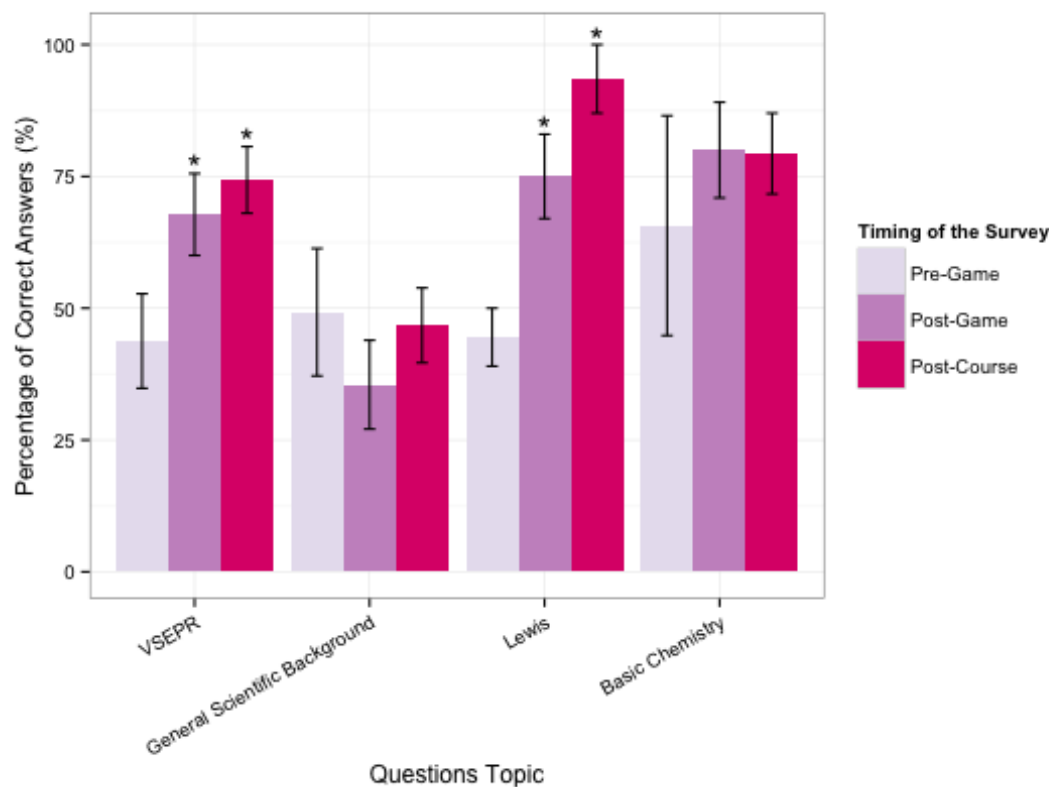


Figure 5.16: Illustration of the evolution of the knowledge of our students before and after playing the VSEPR game (n=18).

The evaluation of the VSEPR game looks promising. For the questions on basic chemistry and GSB that constitute the “control” questions, no significant increase or decrease of the scores of the students are observed. For questions about VSEPR theory, there is a significant increase of the percentage of correct answers obtained after the game and after the course, as compared to the scores obtained prior to playing the game. The increase after the course as compared to after the game survey is not significant, suggesting that most of the knowledge of the students is acquired during the game session.

It is also striking to see the increase of the percentage of correct answers for questions about Lewis after the game and after the course. Again the biggest difference is noticed between the pre-game and post-game survey but it is less clear than for VSEPR questions. The VSEPR game was played one week after the Lewis game. It is clear that to be able to approach 3D issues, the students need to recall and

mobilize their knowledge on 2D representations. That might explain the observed increase.

Long-term evaluation of the Lewis and VSEPR games

After evaluating the direct and short-term benefit of the games on the knowledge of the students, we thought it was important to test the long-term evolution of this knowledge. A survey gathering questions on Lewis and VSEPR was proposed to the students at the end of the first semester (one month after playing the games) and at the beginning of the third semester (around 10 months after playing the games). The survey was answered by the 2012 promotion following this timing. We also proposed this survey to the 2011 promotion but only 12 months after they played the games.

The results of these surveys are shown in figure 5.17. The survey contained 22 questions with 7 qualitative ones and 15 questions testing the knowledge of the students on various topics. The questions were divided into the same 4 categories as for the VSEPR game.

The results of the two promotions are comparable. There is no loss of knowledge by the students on the topics learned through the games. A comparison with courses taught in regular frontal courses would have been valuable.

We can notice a significant increase of the general scientific background knowledge of our students after one semester of courses and two internships. The number of questions (5) was not very large, but it is still encouraging to see this increase.

It is also interesting to compare the actual scores of the students and the way they feel they master a topic. In figure 5.18, we can notice that the students feel like they did not master Lewis theory and representations so well after summer than at the end of the S1. However, their actual answers to questions about Lewis were as correct after summer as at the end of S1. The perceptions of the students about their own knowledge is thus not always very accurate.

5.3.4 Zoom on the Nucleo-Belote Game

The electrophile/nucleophile game, or Nucleo-Belote game, was evaluated by Ana Maria Campos in the University Jorge Tadeo Lozano of Bogotá, Columbia. A pre-game and post-game identical survey was proposed to 24 students involved in a chemistry and biology engineering curriculum. When we met Ana Maria Campos in Paris with Vincent Dahirel, she insisted on the fact that the electrophile and nucleophile concepts were always difficult to learn for her students. She was thus interested to try our game to see if it could ease and improve students knowledge

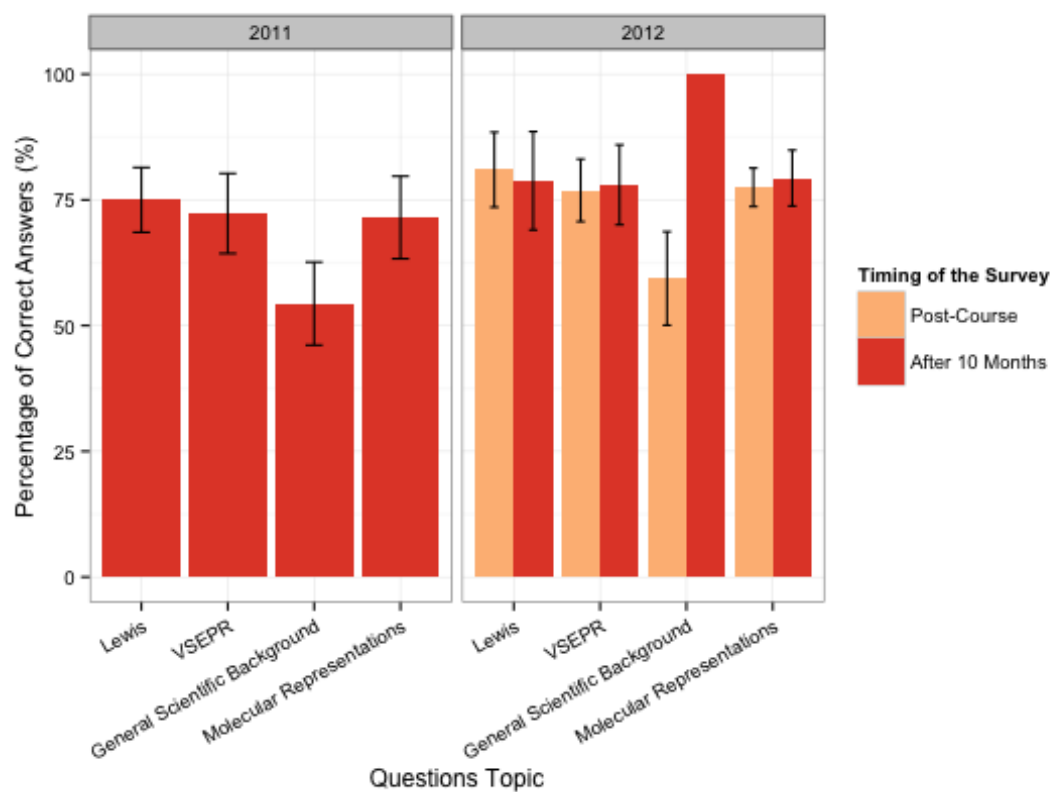


Figure 5.17: Long-term evolution of the knowledge of our students after playing the Lewis and VSEPR games (n=36, two promotions of students).

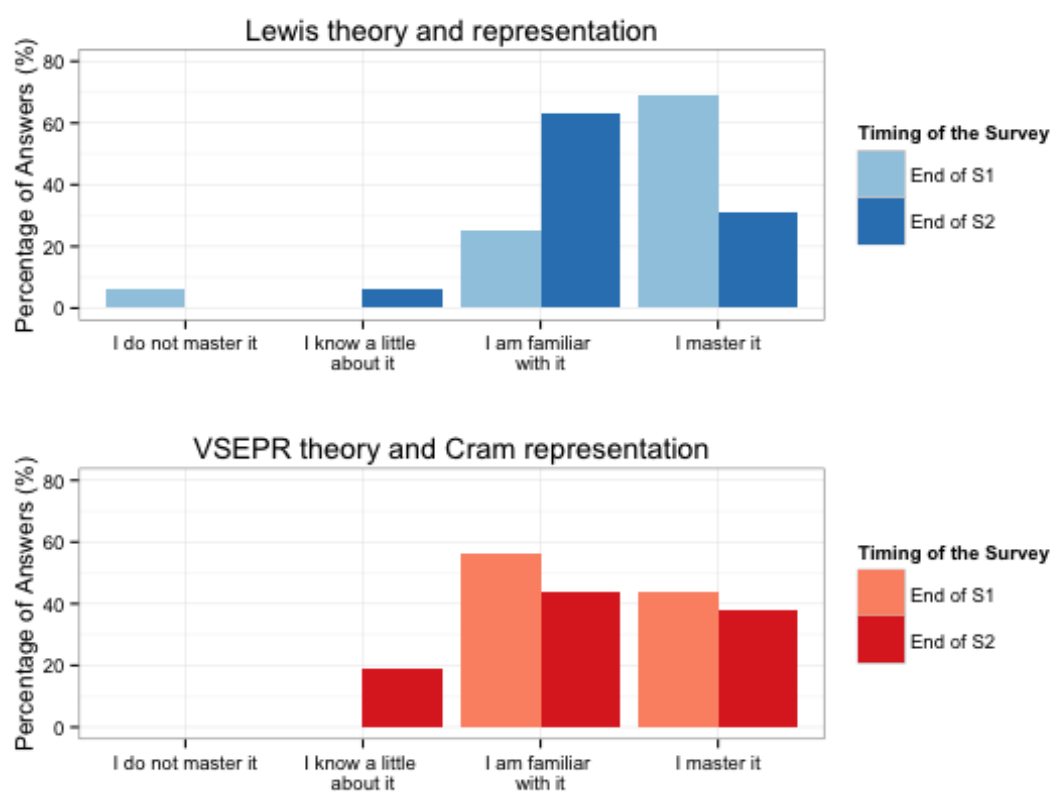


Figure 5.18: Illustration of how much our students felt they knew about Lewis and VSEPR at different time points (n=18).

on these notions.

Ana Maria Campos and her students used the game slightly differently than us. When they used the game, students already learned about basic chemistry concepts such as chemical bond, VSEPR Theory, molecular structure, geometry, electronegativity, and functional groups. The students discovered the electrophile and nucleophile concepts through the game (80% of the students did not know these concepts before playing). When they discovered the cards, they first had to figure out in teams how to differentiate electrophiles from nucleophiles and how to rank them according to their relative reactivity. Then, they imagine rules to play with the cards and played.

The survey proposed to the students contained 9 questions divided into 7 qualitative questions and two questions to evaluate the knowledge of the students respectively on the electrophile and nucleophile concepts.

The main results of the survey were that all the students declared that they enjoyed playing the game and 65% of the students said that they had a lot of fun playing the game (the other 35% of the students had a little fun playing the game). 62% of the students declared that the game helped them to learn what is a electrophile and a nucleophile. This student's feeling was confirmed by the fact that 20% more students were able to recognize a carboxylic acid group as an electrophile after playing the game, and 40% more students were able to tell that a bromide ion is more nucleophile than an alcohol group after playing the game.

Two questions were more open and asked the students to write what they learned through the game and how the game helped them to learn. In summary, they learned:

- The definition of nucleophile and electrophile: "a nucleophile gives electrons and the electrophile is the one that needs electrons".
- To "recognize nucleophiles and electrophiles".
- "The relationship between nucleophiles, electrophiles and electronegativity".
- Predict the relative "power" of an electrophile or a nucleophile.

Some students stressed that it feels easy to learn through the game. Globally the students found that it was more difficult to understand the electrophiles cards than nucleophiles cards. We will thus need to improve our cards to try to improve the balance between the two main families of compounds.

5.4 Discussions and perspectives of the project

5.4.1 Zoom on the periodic table $\chi\varsigma$ game

This game has not yet been properly evaluated with pre-game post-game surveys. This game is usually used as a complement of exercises session after a course that presents the characteristics of chemical elements in accordance to their position in the periodic table.

The feedbacks of the students on this game were good. They said it helped them to memorize both the characteristics of elements and their position in the periodic table. One year this game was used in a slightly different format during several months. At the beginning of each courses a student has to secretly think about a chemical element that the whole class had to guess by asking yes or no questions. This process was quite fast (around 5 minutes) and seemed to be a good exercise for the students.

A similar game as the $\chi\varsigma$ has already been developed online by the Royal Society of Chemistry. However, the number of questions that can be used by the players is limited by the platform. The fact that the machine limits the number of questions and answers is limiting the interest of the game. A multi-player online platform would probably be more appropriate.

Several levels could be imagine for this game with different cards for the chemical elements, showing more or less characteristics. The highest level of the game would use cards that only represent the symbol of the elements.

5.4.2 Further improvements and questions we would like to answer

If the benefit of the use of serious games in teaching is no longer challenged in the community of educational science [5–8], some questions remain open in the literature. Among these questions, we have chosen to address some that seem particularly adapted to investigate with our games, our student audience, and in the learning environment of the CRI.

First, we would like to study further the pedagogical impact of the games on the notions approach by them. For example, we would like to answer the following questions:

- How much games allow the students to understand, memorize and consolidate in the long-term knowledge and know-how?

- Are games useful to consolidate concepts previously learned?
- What are the complementary and relative benefits of a learning through games approach as compared to a classical frontal lecture?
- Are there differences between games that are used to learn vs to consolidate new concepts?

To answer thoroughly this questions we will need to improve our surveys (by working on the balance between the different categories of questions and on the level of difficulty of the questions) and design experiments with proper students control groups.

We also would like to examine the interactions between the social and “scholar” background of the students and the way they learn through the games. We can wonder if students coming from an artistic curriculum that foster more their imagination, or from a more practical curriculum, might be more open to this pedagogical approaches. For example we could formulate the question:

- Are there specific psychological traits or socio-cultural contexts that lead some students to benefit more from the learning through games approach than others?

The next field to investigate touch the way students perceive their knowledge and its evolution. We can wonder if the games help the students to feel more confident or if it ease the learning process as it was suggested by some of the students who played the Nucleo-Belote game. One interesting question would then be:

- What is the difference between the self-perception and the actual knowledge of the students, and is this difference depending on the type of pedagogy used?

Finally, beyond the chemical concepts and competences learned through the games, the students also develop soft-skills while playing. They learn to work in teams and they also develop their imagination and foster their creativity. These outcomes of the games have not yet been tested but we would be very interested to include them in our future studies.

In the case of the two inquiry games on Lewis and VSEPR, we believe that the games recapitulate the steps of a experimental research method. Students first need to observe available data and draw hypothesis based on them. They can then do “further experiments” (ask for new cards or ask for advices) to test these hypothesis and make them evolve till they explain all the observations that they gathered. Thus we would like to test the question:

- Are the Lewis and VSEPR games useful to approach and understand experimental research reasoning?

5.4.3 Involving students and teachers in the evolution of the games

To answer all these questions and develop our project we need to find more partners and collaborators and involve more people in the project.

Our students were very curious and volunteer to get seriously involved in the pedagogical study of the games. Some students already participated in a week of brainstorming and improvement of the games. During this intensive week of internship with them, we studied some literature, clarified and improved the rules of our games and developed two new games about organic chemistry (see the next section). The week ended by a chemistry game night open to all curious people, scientist or non-scientist, kids or grown-ups, who wanted to test our games and give us feedbacks on them. The results of the surveys have not yet been analysed but the feedbacks we got on the spot were very interesting and encouraging.

At the occasion of this intensive week we opened a blog on the project and tried to contact chemistry teachers to get them involved in the project. It would be very interesting for us to have more insights into the notions that are really difficult to grasp by the students to try to insist on them in our games. This networking process will need to be pursued and reinforced.

Finally, the students of the FDV bachelor program could be involved more systematically in the studies. The statistical analysis of the results collected by the surveys could for example be a good teaching material for their statistics courses.

5.4.4 New games in construction about organic chemistry

The two games presented below started on my impulse but were considerably clarified and developed during the intensive week that we organized for some volunteered students of the FDV bachelor program. The first game about the mechanisms of organic reactions was already tested twice in our classrooms at a very “draft” stage. The second was only tested during the chemistry game night open to the general public at the end of the intensive week.

A game to build mechanisms of reactions

this game prolong the Nucleo-Belote game. It fosters knowledge on the reactivity of the molecules. In addition it introduces notions about reactions mechanisms and about the influence of experimental conditions on the mechanism of a reaction.

The rules of this game are inspired by the game “Alhambra”. In this game players trade currencies and place tiles representing buildings to construct an Arabic palace.

The chemistry version of the game is a little more complex. The aim of the players is not to build a palace but to build a chemical reaction and its mechanism. For this purpose they need to buy the reactants of the reactions as well as the main reaction intermediaries. The money are atoms. To buy a given molecule you need to have the good number of atoms of each type contained in this molecule. During each round of the game the players can in turns buy and/or position the molecule they previously bought on their board that represents the different steps of the reactions. Molecules with low reactivity are easier to buy than highly reactive species. Nevertheless, at the end of every two rounds, each player can choose a special power in a set of proposed special powers. A special power can be for example that you can use a very polar solvent, or a protic solvent, that you can use an acid or basic catalysis, that you can increase or decrease the temperature...etc... This way the students can turn a less reactive specie into a very good deal of they choose and use their special powers appropriately.

At the end of the games the students need to draw the complete mechanisms of the reactions they built and give the major products. If the mechanism and the product is right, they get points for this reaction. The student with the most points win the game.

This game was positively perceived by our students. They had fun and enjoyed the special powers part that triggered a lot of discussions among them. One of the limitation of this game in its current format is that it is quite long (around 40 minutes) and probably more interesting in terms of learning outcomes if you play it several times. One possibility to overcome this limitation is to improve the mechanisms of the game so that it is really fun and the students enjoy playing it outside of the courses hours.

The “Cluedo” of organic reactions

This game was created to go further in the study of the influence of the experimental conditions on reactions and to introduce notions about competitive reactions. This game mobilizes as well the knowledge of the students on analytical chemistry and spectroscopy.

The rules of this game are inspired by the game called “Cluedo”. This game always begin with the description of a crime scene. The goal of the players is then to figure out who committed the crime and with which weapon. In the chemistry version of the game, the victim is one of the reactant of a chemical reaction who was

transformed into an unknown different specie (fig. 5.19). The goal of the players is to figure out who was the culprit of the transformation, that is to say find the other reactant of the reaction, and into which specie the victim was transformed, that is to say find the product of the reaction. The players also need to discover the experimental conditions of the reaction and place their piece into the good “bescher” to announce the names of the novel specie and of the culprit.



Figure 5.19: Picture of the set up of the “Cluedo” of the reactions game, developed with students from the FDV bachelor (namely Ivan Cornut, Quentin Ferdinand, Ioana Genescu, Juliette Lenouvel, Emeline Oueda-Cruz, Rebecca Richard, Pierre-Luc Satin).

To be fun the game need to be based on reactants that are susceptible to give many different products depending on experimental conditions. The first version of the game was built on the competitive reactions arising from the alcohol family (dehydration, formation of a carboxylic acid, oxydation or reduction...).

To discover the novel specie formed and the second reactant of the reaction, the players can visit some laboratory and get results from experiments done on one or the other of the unknown molecules. They can get for example the infra-red or

NMR spectrum of a molecule, the boiling point of a molecule, or results of radioactive marking experiments to trace the changes happening to the victim.

This game was only tested during the general public chemistry game night. The level of the game was clearly not adapted to non-scientist but I think the students who participated in the development of this game, learned a lot about chemistry of organic reactions. In general, it is very valuable to involve the students in the evolution of the rules of our games. Through this process they have to zoom out and integrate different chemistry concepts.

5.4.5 From board games to digital online games

The board and card games developed in this project can be transformed into digital games. This transfer will open new perspectives. Digitalization would allow the students to play alone, out of the classroom. More generally, every game could be played by a broader public, outside of a formal teaching context. In addition, the digital environment allows to create more complex games that could be more challenging. Finally, our pedagogical content can easily be connected to the video-gaming experience, through links to videos of courses or educational websites.

For the Lewis and VSEPR games and the periodic table $\chi\varsigma$ game, the content of the future digital games will be very similar to the board games. For the games on organic chemistry, our goal is to greatly extend the set of organic reactions involved and to offer a significantly wider experience that would immerse the players in a simulated chemistry lab. We imagine a game where players, alone or in teams, are chemists evolving in a virtual laboratory. To progress in the game, players will learn many concepts of organic chemistry: reactivity of molecules, the role of the solvent, the role of the temperature in the kinetic of reactions, safety issues, environmental impact, ...etc. The game will be progressive to allow novices in chemistry to gradually learn about organic chemistry, while encouraging players who know more about chemistry to progress faster to investigate problems of complex reaction dynamics. The goal of chemists or team of chemists in the game will be to produce more and more complex molecules with higher yields.

Just like the board and card games, this online game will be co-constructed with the help of students and teachers. In addition, the whole community of players will contribute to enrich the catalog of chemical reactions of the game, offer new way of synthesis, warn about the environmental impact of some reactions.

In parallel, we would like to create a digital platform around these games, where the players who wish may find courses explaining the algorithms underlying the game, and the chemical theories supporting these algorithms. These courses will be

presented either as videos or written online posts. In addition to these courses, a whole set of related documents will be available: scientific publications describing the reactions used in the game (kinetics of reactions, energy of reactions), chemicals safety database.

In conclusion, this chapter probably contains more perspectives than significant results but it presents an exciting project and a beautiful human adventure that will probably lead us to many surprises and original discoveries.

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Global Conclusions and Perspectives

...Integrating always more inputs to build innovative projects...

To my point of view the main scientific achievements of my PhD are the preliminary reconstruction of a three-population *in vitro* oriented neuronal network and the development of the fully optical method to stimulate and record neuronal activity. I believe that combining these two technologies can lead to many more beautiful discoveries in the future. Building a full cortex-hippocampus network *in vitro* and observing its dynamics would be really interesting and it would give new perspectives to the emerging field of *in vitro* cognitive studies.

The study on micro-patterns is a very exciting biophysical study but I must say that, for the moment, I think the main achievement of this part of my work is the development of the analysis method using the Fourier Transform. To be sure of the interest of the biophysical results presented in this manuscript, I would like to collect more data to improve the image database quality. I am quite confident that the biological results we will then get, can be surprising and powerful in many ways.

The work I did in pedagogy to teach chemistry through game is still preliminary. This study is important for me because my colleagues in this adventure are very close friends. And if there is one thing I learned, beyond science, during my PhD is that I work much better and with much more pleasure when I work with people I trust and I appreciate, not only for their intellectual capacities but mainly for their human and social values. The project we did with Renaud Renault is also, to my view, a good example of this winning combination.

Between *in vitro* neuronal studies and pedagogy there seem to be a big gap. However, in both cases, we approach cognitive processes from unusual point of views. The bridge between the two part of my PhD can probably be found in the field of cognitive neuroscience. Some links already exist between educational science and

cognitive studies, they are still under constant debate. very few links exist between cognitive science and *in vitro* neuronal studies probably due to an ancient cleavage between *in vivo* and *in vitro* biologists, and maybe also because of our fear to be able to reduce our cognitive capacities to simple biochemistry and biophysical mechanisms. Nevertheless, more and more scientists dare talking about cognitive processes happening *in vitro* and I think we are not so far from building an “In Vitro Artificial Intelligence”.

I dream about a time where these three disciplines merge. We would then be able to draw hypothesis from observations in the classrooms about how students learn, transpose these hypothesis to test them in a cognitive science laboratory and finally test them in a “petri dish”. We would then be able to go back in the classroom and improve our teaching methods to ease learning processes. Will that research world stay a dream? I hope not to have the pleasure to participate in it.

Future scientific projects

For a first post-doctoral position, I will join the group of Karen Martinez (Bionanotechnology laboratory of the Nano-Science Center of the University of Copenhagen). During my post-doctoral stay, I would like to combine the knowledge and techniques I acquired during my PhD thesis with the innovative nano-technologies available in this new laboratory. My goal in this project is to use vertical arrays of NanoWires (aNWs) to study *in vitro* the formation, chemical activity and plasticity of single synapses in neuronal networks with controlled topologies.

In our brains, synapses are the transmission points between neurons and their dynamics allow us to think, learn and memorize. At single synapses, diverse molecules, called neurotransmitters, are released and transmit information from the pre-synaptic to the post-synaptic neuron. Glutamate and GABA are among the most abundant neurotransmitters with respectively excitatory and inhibitory effect on the post-synaptic neuron. Individual synapses can modulate their efficiency by varying the nature and amount of neurotransmitters they release. A deeper understanding of these phenomena requires investigations at the single synapse level. Until now, synapses have been intensively studied *in vivo* where the physiological complexity limits the investigations at this level. There remain needs to (1) better understand the mechanics of synapses formation; (2) observe directly the chemical activity at single synapses; (3) investigate the role of neuronal network topology in synaptic plasticity. Alternative *in vitro* approaches, using recent advances in microfluidics and nanowires technology, now open new routes to overcome classical limitations that I would like to explore.

*“Je suis superficielle, et fière de l’être,
parce que la superficie que je couvre est immense !”
Sophy Caulier*

Chapter A

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PAPER

Axon diodes for the reconstruction of oriented neuronal networks in microfluidic chambers†

Jean-Michel Peyrin,^{‡*a} Bérangère Deleglise,^{‡a} Laure Saias,^{‡§c} Maéva Vignes,^{ac} Paul Gougis,^c Sebastien Magnifico,^a Sandrine Betuing,^b Mathéa Pietri,^a Jocelyne Caboche,^b Peter Vanhoutte,^b Jean-Louis Viovy^{*c} and Bernard Brugg^{*a}

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Various experimental models are used to study brain development and degeneration. They range from whole animal models, which preserve anatomical structures but strongly limit investigations at the cellular level, to dissociated cell culture systems that allow detailed observation of cell phenotypes but lack the highly ordered physiological neuron connection architecture. We describe here a platform comprising independent cell culture chambers separated by an array of “axonal diodes”. This array involves asymmetric micro-channels, imposing unidirectional axon connectivity with 97% selectivity. It allows the construction of complex, oriented neuronal networks not feasible with earlier platforms. Different neuronal subtypes could be co-cultivated for weeks, and sequential seeding of different cell populations reproduced physiological network development. To illustrate possible applications, we created and characterized a cortico-striatal oriented network. Functional synaptic connections were established. The activation of striatal differentiation by cortical axons, and the synchronization of neural activity were demonstrated. Each neuronal population and subcompartment could be chemically addressed individually. The directionality of neural pathways being a key feature of the nervous system organization, the axon diode concept brings in a paradigmatic change in neuronal culture platforms, with potential applications for studying neuronal development, synaptic transmission and neurodegenerative disorder such as Alzheimer and Parkinson diseases at the sub-cellular, cellular and network levels.

Introduction

The brain is an exquisitely complex structure, comprising many different interconnected neuronal areas in which each neuron makes hundreds to thousands of specific connections with nearby and remote cognate neurons. Various experimental models are used to study brain development and/or degeneration. These models range from whole animal models, in which the anatomical structures are

intact but experimentation at the cellular level is severely restricted, to dissociated cell culture systems allowing fine observation of cell phenotypes but lacking the highly ordered connectivity of neurons in the brain. *In vitro* studies of neuronal connections have generally been limited to observations of small numbers of cells in dissociated two-dimensional (2D) cultures with random connections, or in slice cultures, in which connections follow 3D intermingled networks that vary from one sample to the other, and are partly severed by the slicing process. In addition, these standard cell culture systems cannot be used to study the spatial compartmentalization of neuronal signals. Two major technical issues have been hampering the elucidation of axonal and synaptic differentiation and degeneration: first, a separation of soma from neuronal endings is a prerequisite for studying local molecular mechanisms in different sub-cellular compartments; second, it was not possible so far to create oriented neuronal networks involving different neuronal subtypes.

Microfluidic techniques, exploiting microfabrication technologies and the physical properties of flows at the micron scale, are powerful tools for studying living cells.¹ Notably, microfluidic devices allowing the physical separation of soma from axons, thanks to micro-channels, were used to study neuronal cell behavior.^{2–4} This type of system allows

^aLaboratoire de Neurobiologie des Processus Adaptatifs, CNRS, UMR7102, Université Pierre et Marie Curie, Paris, France. E-mail: jean-michel.peyrin@snv.jussieu.fr; Fax: +33 1 44 27 26 69; Tel: +33 1 44 27 32 42; bernard.brugg@snv.jussieu.fr

^bPhysiopathologie des Maladies du Système Nerveux Central, CNRS, UMR7224, INSERM UMR5 952, Université Pierre et Marie Curie, Paris, France

^cMarcomolecules and Microsystems in Biology and Medicine, Institut Curie, Centre National de Recherche Scientifique, Université Pierre et Marie Curie, UMR 168, 75005 Paris, France. E-mail: jean-louis.viovy@curie.fr; Fax: +33 1 40 51 06 36; Tel: +33 1 56 24 68 45

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‡ Authors equally contributed to the work.

§ Current address: Cell and Developmental Biology, Centre for Genomic Regulation, 08003 Barcelona, Spain.

pharmacological intervention focused on axons or neuronal somas of a single neuronal subtype. However, accurate reconstruction of oriented neuronal networks with a connectivity similar to that formed *in vivo* by neurons originating from distinct brain structures was not possible because neurons seeded in both cell culture chambers send their axons to the respective opposite chamber.⁵

Here, we propose a microfluidic system allowing the reconstruction of oriented neuronal networks involving several different neuron populations with a predefined connection architecture and a specific fluidic access to various cellular subcompartments within these networks. The key innovation of this system is the presence of asymmetrical micro-channels that can be penetrated by axons in a single direction, separating two individual chambers (or more). This asymmetrical geometry operates as a directionally selective filter for axons and as an impermeable barrier for cell bodies. It functions as a “diode” for axonal projections. We seeded primary neuronal cultures in this directional system, reconstituted cortico-striatal projections, and demonstrated the formation of active neuronal connections. *In vivo*, the striatal neurons present in the neostriatum are the principal recipients of glutamatergic cortical afferents (fibers of axons issued from cortical neurons) from the basal ganglia.^{6,7} Using immuno-cytochemistry and functional analysis we showed that cortical fibers trigger full differentiation of medium spiny neurons (neurons contained in the receiving chamber, which were only partly differentiated before the arrival of cortical axons), with extensive formation of dendritic spines (a morphological signature of neuronal connections) and functional glutamatergic cortico-striatal synapses. This leads to the creation of an integrated binary neuronal network that fully mimics the *in vivo* neuronal pathway, opening the route to a number of applications ranging from developmental neurosciences to neuropharmacology. We finally discuss the generality of this platform and its potential for studying localized degenerative events at the cellular and subcellular levels in various neurodegenerative diseases, or for studies of physiological brain development and neuronal communication.

Results

Set up of an oriented microfluidic cell culture system allowing directional axonal outgrowth

Our microfluidic neuron culture platform involves two main features. The first one is the culture of two (or more) cell populations in microfluidic chambers, connected by an array of microstructures allowing axonal communication between these chambers without mixing soma. This system was inspired from Taylor *et al.*³ The second one is a new and original approach to reproduce the directionality of neuronal connections observed *in vivo*, a critical parameter for efficient neuronal functioning. Previous studies have shown that axonal growth can be controlled by mechanical constraints^{8,9} and that axons tend to follow straight microfluidic structures, a property previously used by Jeon's Lab³ to separate, in two distinct chambers, axonal endings from neuronal somas (Fig. 1a). In an effort to optimize the microstructures allowing crossing and invasion of the axonal chamber, we used a multi-dimensional chip comprising 2 distinct cell culture chambers separated by arrays of rectangular micro-channels of decreasing width (Fig. 1b). As shown in Fig. 1c, the number of axons per channel is highly correlated to the width of the channels. Whereas large micro-channels, 20 to 15 μm wide, allow a complete invasion of the distal chamber with 15 to 20 axons per channel, decreasing the width reduces the number of axons entering the channels. For really narrow ($<3\text{ }\mu\text{m}$) and long micro-channels axonal length and integrity are reduced. Moreover we observed that axons encountering a wall perpendicularly deviate, whereas axons encountering a wall tangentially continue growing along the wall.

We tried to exploit these properties to impose a directionality that would allow axons to grow only from one chamber (emitting) to the other (receiving) and not the opposite way. Based on the effect of width of rectangular micro-channels on axonal crossing (Fig. 1b), we designed asymmetrical funnel-shaped micro-channels (named “axon diodes”). The resulting oriented microfluidic cell culture comprises two distinct cell culture chambers (Fig. 2a), each connected to two reservoirs and

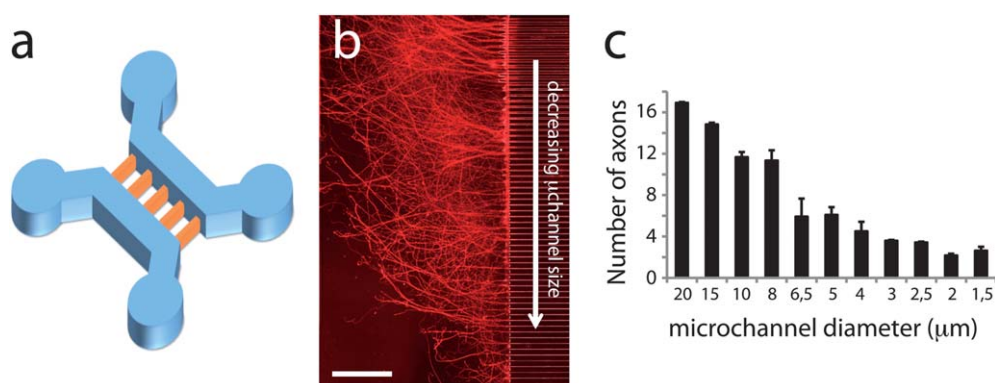


Fig. 1 Effect of the width entrance of rectangular straight channels on axonal crossing. (a) The microfluidic neuronal culture devices derived from ref. 3 are composed of two separate cell culture chambers separated by an array of rectangular micro-channels (500 μm long, 3 μm high) with eleven variable width ranging from 20 to 1.5 μm by groups of 6 identical micro-channels per width. (b) Primary cortical neurons seeded in a multidimensional microfluidic chip with variable width ranging from 20 to 1.5 μm . 10 days after cell seeding, the axonal chamber was immuno-stained for tubulin. (c) Quantification after 10 days of culture, of the number of axons exiting the micro-channels.

separated by a series of 500 μm long, 3 μm high, asymmetrical micro-channels (Fig. 2b). Based on Fig. 1c we chose a 15 μm entrance allowing optimal axonal collection from the emitting chamber. As shown in Fig. 2c, when seeded in the chamber on the wider (“emitting”) side of the axon diodes, mouse primary cortical neurons emit axons; their entrance into the micro-channels being facilitated by the large openings. Axons that have entered a micro-channel tend to fasciculate and grow in bundles; they do not stop in the narrowing section, guided by the channel walls. In order to assess whether the funnel like channel causes axonal “engorgement”, we then tested the influence of the narrowest side width on axonal crossing. In comparison to a 15 μm wide rectangular channel, 15 to 5, 3 or 2 μm wide diodes allowed 90%, 70% and 30% respectively of receiving chamber invasion (not shown). Using a 15 to 3 μm diode, 8 days after cell seeding, the “receiving” (narrow side of the channel) chamber is massively

invaded by axons which de-fasciculate and branch 50 to 100 μm after exiting the micro-channels (Fig. 2c).

In contrast, when neurons are seeded at the same density in the “receiving” chamber, the number of axons reaching the opposite chamber is greatly reduced (Fig. 2d). Axons tend to bump into the sidewall of the cell culture chamber and keep their new directionality by following the wall of the receiving chamber without entering the narrow micro-channels (only a small fraction is able to enter the micro-channel “tips”). The relative amount of axon projections from both sides was quantified for an optimal width of micro-channel tips. For 15 μm wide openings on the emitting side and 3 μm wide on the receiving one, the number of axons projected with the “forbidden” polarity (*i.e.* from the narrow side to the wide side) was only around 3 to 5% of the number of axons projected along the allowed polarity (Fig. 2e).

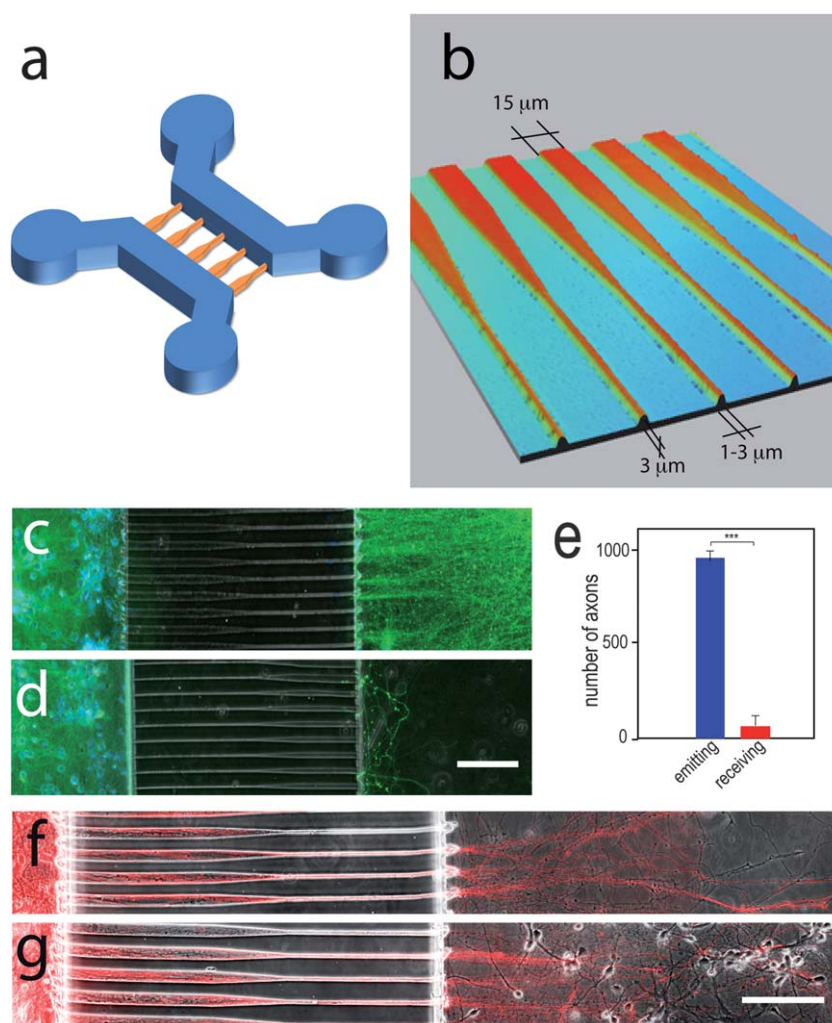


Fig. 2 *In vitro* reconstruction of a neuronal network using “axonal diodes” in microfluidic culture devices. (a) Global 3D view of the microfluidic device comprising asymmetrical microchannels (b) 3D view of the funnel-shaped micro-channels as observed by white-light optical profiling (WLOP) with an interference microscope. Immunofluorescent images of microfluidic cultures (green: α -tubulin, blue: Hoechst) in which cortical neurons were seeded either on the wide (c, 15 μm) or the narrow (d, 3 μm) side, at a concentration of 45×10^6 cells per ml. Note the lack of labeling in the wide chamber when cortical neurons are seeded in the narrow side (d). (e) Quantification showing that the axonal diodes efficiently polarize axonal growth (***) p -value < 0.001). Phase contrast image combined with epifluorescence of a reconstructed polarized neuronal-network after 8 days *in vitro*. To visualize individual axons of cortical neurons, cells were transduced with a m-cherry Sindbis viral vector (red). Cortical neurons projecting their axons into the second chamber without a target (f) or in the presence of striatal neurons seeded in the second chamber (g). Note that striatal neurons are not infected by the virus, indicating an effective compartmentalization of the two culture chambers. Scale bar 50 μm .

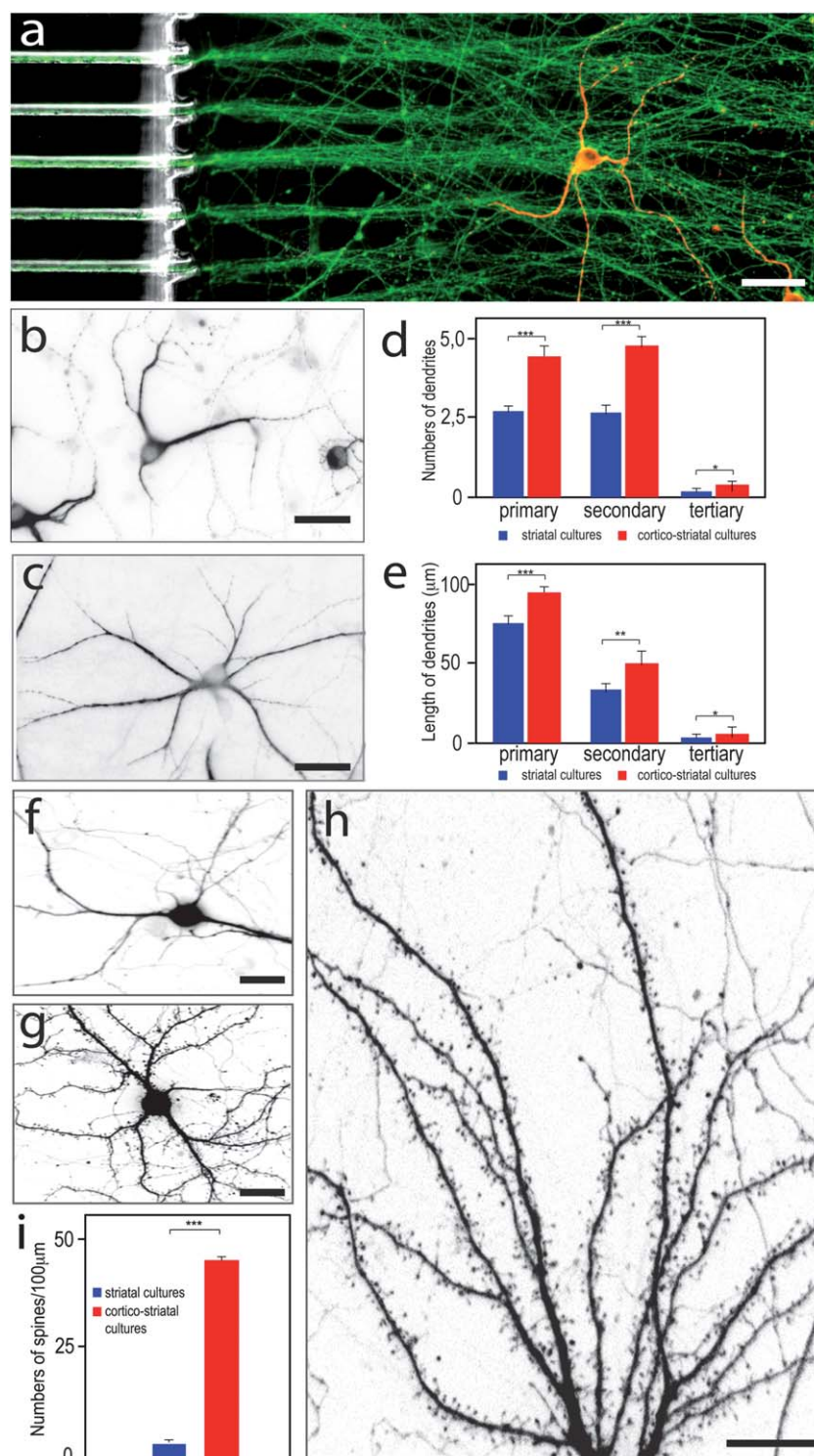


Fig. 3 Cortical afferents promote striatal neurons differentiation. (a) Dendritic MAP-2 immuno staining (red) of striatal neurons (13 DIV) receiving cortical afferents (in green, α -tubulin). Morphological analysis of striatal neuron arborization using MAP-2 immunolabeling in the absence (b) or presence of cortical afferents (c). Quantification of the number (d) and length (e) of striatal neuron dendrites. (d) p -Value: primary = 5.5×10^{-4} , secondary = 5.5×10^{-3} , tertiary = 9.6×10^{-3} . (e) p -value: primary = 3.9×10^{-5} , secondary = 2.1×10^{-5} , tertiary = 4.6×10^{-2} ($n = 40$ neurons per condition (3 independent experiments)). (f–h) Images of GFP-expressing striatal neurons. Striatal dendrites receiving afferents from cortical neurons have many more dendritic spines (g and h) than those cultured alone (f). (i) Quantification of spine density (per 100 μm) in GFP positive striatal dendrites grown in the presence and absence of cortical afferents (** p -value < 0.001). Scale bar: 50 μm .

Establishment of an oriented neuronal network

We then reconstructed oriented neuronal cortico-striatal projections. Primary cortical neurons from the neocortex were seeded on the emitting side of the diodes, and striatal neurons from the same mice were seeded on the receiving side. Immunocytochemical analysis shows that cortical cells are composed of 90% MAP2 positive neurons among which 5 to 10% are GAD67 inhibitory interneurons and around 90% are VGLUT1 glutamatergic neurons. After two weeks in culture, striatal cells seeded in the receiving chamber are composed of 80% neurons among which 90% are GAD67 positive inhibitory neurons (ESI, Fig. S2†). Fig. 2f shows phase contrast micrographs of cortical neurons projecting their axons through the axonal diodes and connecting to the striatal neurons in the receiving chamber after 8 days in culture (Fig. 2g). To distinguish cortical axons growing in the striatal chamber from striatal axons and dendrites, cortical neurons were transduced after 7 days in culture in the emitting chamber, with a Sindbis viral vector encoding m-cherry fluorescent protein. The viral vector transduced up to 80% of cortical neurons. Their axons projecting in the receiving chamber were stained in red and easily discriminated from unlabeled striatal axons and dendrites (Fig. 2f and g). These axons established contacts with striatal neurons and continued to grow across the receiving chamber suggesting the formation of “en passant” connections as those observed *in vivo* in the cortico-striatal pathway. The oriented network in the diode devices was maintained routinely for 3 weeks *in vitro*, thus allowing short and long-term experiments.

Cortical fibers establish synaptic contacts with striatal dendrites and induce spinogenesis and striatal maturation

Having shown that cortical axons invade the receiving chambers we tested whether the presence of cortical fibers in the receiving chamber affected striatal differentiation. After 15 days *in vitro*, cells were fixed and immunostained for MAP-2 to evaluate the degree of striatal neuron arborization (Fig. 3a). Pure striatal cultures present many immature-like neurons, as compared to the numerous mature-like multipolar neurons with extended processes found in cortico-striatal co-cultures (compare Fig. 3b and c). Morphometric analysis on striatal neurons grown alone or in the presence of cortical afferents showed that the former had in average 3 primary (starting from the soma) and secondary dendrites (starting from primary dendrites) and no tertiary dendrites per cell, whereas the latter had in average 4 primary, 5 secondary and 1 tertiary dendrites after 2 weeks in culture (Fig. 3d). The dendrites of striatal neurons grown in the presence of cortical axons were also significantly longer (Fig. 3e). Interestingly this differential maturation is exacerbated after 3 weeks in culture as shown in Fig. 3f–g. Another morphological consequence of the presence of cortical afferents on the formation of striatal dendritic spines was revealed by culturing striatal neurons from transgenic mice expressing GFP (green fluorescent protein) under the control of an actin promoter in all cell types. GFP positive striatal dendrites grown in the presence of cortical afferents had many dendritic spines (40 spines per 100 μm) whereas striatal neurons cultured alone were virtually devoid of spines (2 spines per 100 μm) (Fig. 3f–i). These findings indicate that cortical fibers coming from the emitting chamber promote striatal differentiation *in vitro* in the receiving chamber.

To confirm that cortical fibers form synapses on striatal dendrites, neurons were stained for the pre- and postsynaptic markers VGLUT1 and PSD-95, respectively. Whereas both VGLUT1 (Fig. 4b) and PSD-95 (Fig. 4d) show faint and sparsely clustered signals on unconnected cortical fibers or single striatal neurons, respectively, cortico-striatal projections (Fig. 4a and c) led to a dramatically increased labeling of the presynaptic markers along cortical axons, with dense clustering of VGLUT1 (Fig. 4a, inset a'), and clustering of the post-synaptic marker PSD95 (Fig. 4c, inset c' and c'') along the MAP-2 positive striatal dendrites. Co-localization experiments showed that presynaptic cortical VGLUT1 staining is present on top of the GFP positive striatal dendritic spines apposing to postsynaptic PSD-95 positive striatal structures (arrow heads, Fig. 4e).

Functionality of the cortico-striatal synapses

We next assessed whether the synapses of the cortico-striatal network were active. Striatal neurons are GABAergic neurons expressing various glutamate receptor subtypes including members of the NMDA receptor subfamily. Electrical stimulation of the glutamatergic cortico-striatal pathway triggers a phosphorylation cascade leading to the activation of the Extracellular-signal Regulated Kinase (ERK).¹⁰ Immuno-cytochemical detection of phosphorylated ERK (P-ERK) in striatal neurons shows that approximately 15% of striatal neurons cultured alone exhibit robust P-ERK cytosolic staining (Fig. 5c). However P-ERK staining increases to 30% when grown in contact with cortical fibers, indicating that cortico-striatal connections induced a basal ERK activation (Fig. 5a and b). When the cortical emitting compartment was stimulated with KCl, to depolarize cortical neurons, the proportion of striatal neurons showing P-ERK staining increased to 60% (Fig. 5c). This activation was partially inhibited by incubation with the glutamatergic NMDA receptor (NMDAR) antagonist MK801, within the receiving striatal chamber (Fig. 5c). A Fluo4 non-ratiometric calcium analysis of primary neurons (Fig. 6) revealed that cortical neurons progressively show spontaneous slow calcium oscillation reminiscent of intrinsic cortical connectivity (Fig. 6a–c). After 12 to 16 days *in vitro*, approximately 50 to 70% of cortical neurons exhibit synchronous calcium oscillation (Fig. 6c), while when grown alone, striatal cells never show any sign of calcium oscillation (Fig. 6d). In contrast, striatal neurons connected to cortical fibers acquire spontaneous slow calcium oscillation in synchrony with cortical fibers (Fig. 6e), a process abolished by striatal MK801 (Fig. 6f) or TTX (Fig. 6g) application.

In summary, our system allows the reconstruction of an oriented and fully mature cortico-striatal neuronal network, in which post-synaptic neurons are functionally connected to glutamatergic cortical fibers.

Discussion

Advantages of microfluidic axonal diodes for neuronal network reconstruction *in vitro*

During the last few years, microfluidics has brought new perspective in the development of models in cell biology. Several systems have been proposed to reconstruct neuronal networks.^{11–14} Relatively

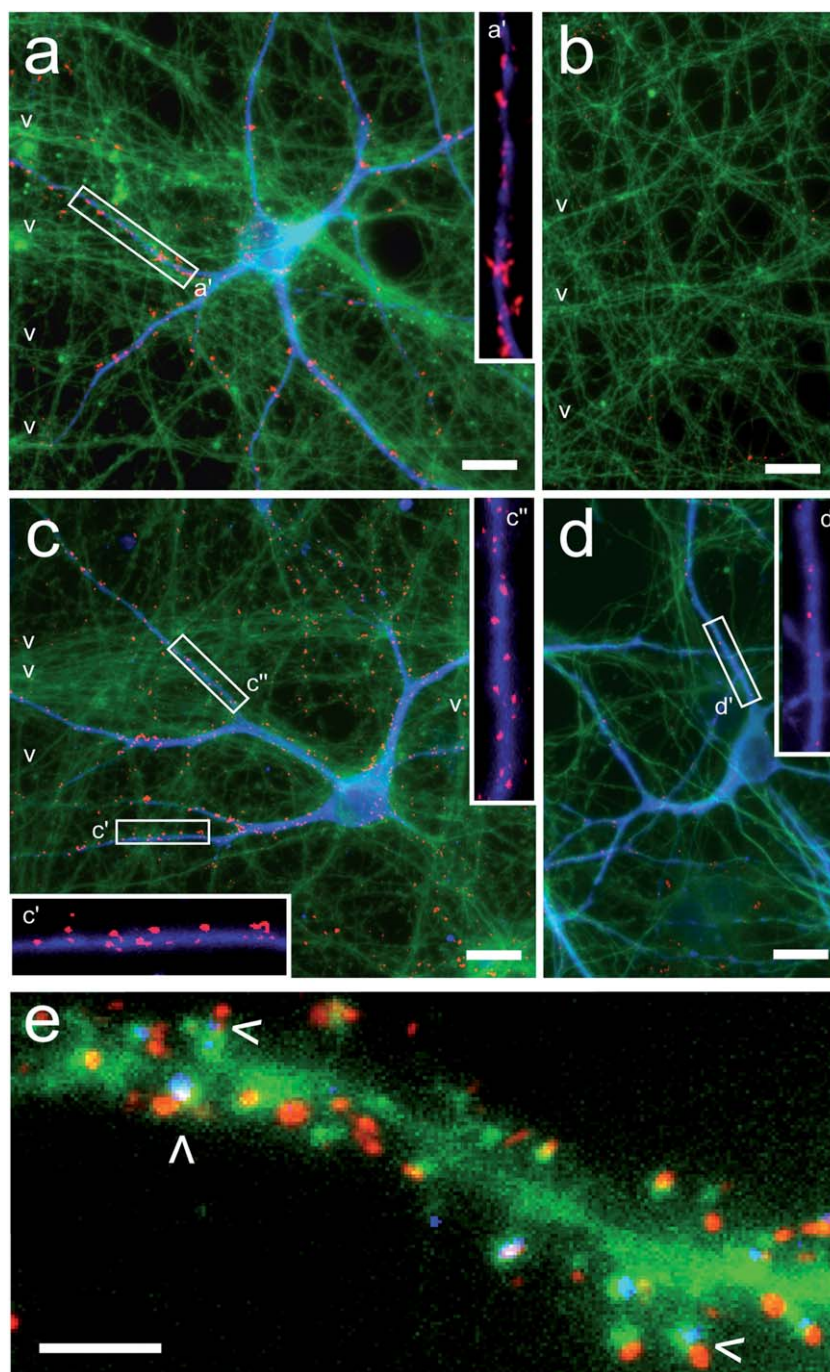


Fig. 4 Cortico-striatal microfluidic co-culture enhances synaptic maturation. (a and c) Striatal cultures in the presence of cortical fibers. Neurons in the striatal chamber were immunocytochemically labeled for MAP-2 (blue), and afferents from cortical neurons for α -tubulin (green, arrowheads). VGLUT1 (red) (a and b) and PSD95 (red) (c and d) were used as pre- and post-synaptic markers, respectively. (b) Cortical axons cultured in the absence of striatal neurons. Note that cortical neurons present scarce and dispersed VGLUT1 clusters on their axons, reminiscent of partial maturation of presynaptic terminals. (d) Striatal neurons cultured alone present sparse clusters of PSD-95 indicating an intermediate state of differentiation of striatal neurons. The greater VGLUT1 (a) and PSD95 (b) staining and clustering are observed especially where cortical axons and striatal neurons seem to connect, reminiscent of synaptic protein relocalization to cortico-striatal contacts. a', c', c'' and d': higher magnification of the synaptic markers (red) on striatal dendrites (blue). Co-localization experiments showed that presynaptic VGLUT1 staining is present on top of the GFP positive striatal dendritic spines apposing to postsynaptic PSD-95 positive striatal structures (e). Scale bar: 10 μ m.

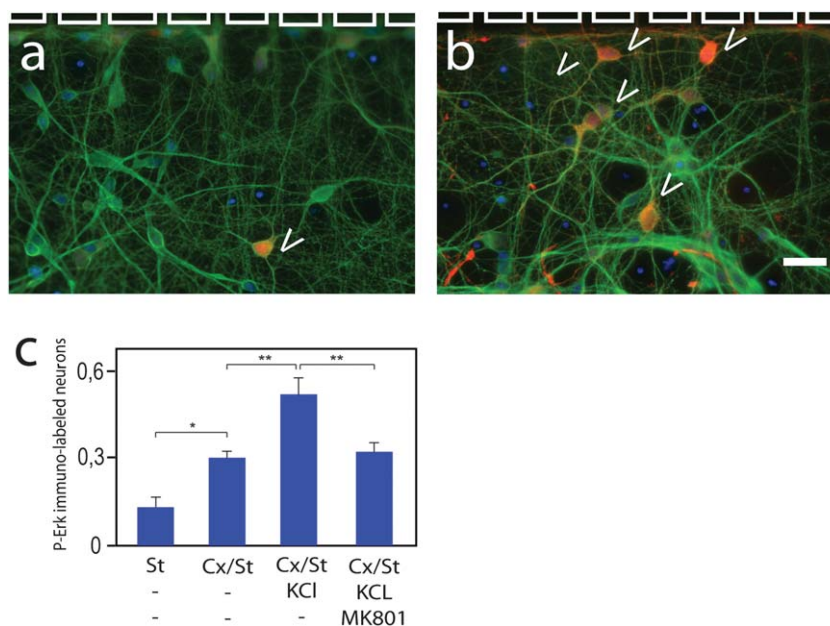


Fig. 5 Cortical depolarization induces glutamatergic neurotransmission and subsequent ERK activation. Cortico-striatal networks were grown for 12 to 14 days *in vitro*. ERK activation was analyzed in striatal neurons by immunocytochemical detection of its phosphorylated form (P-ERK, red): P-ERK labeling in striatal neurons (arrowheads) in basal conditions (a) and 20 min after a stimulation of the cortical somato dendritic compartment with KCl (50 mM) (b). (c) P-ERK signal was counted in 3 independent experiments before and after KCl treatment, in the presence and absence of MK801, a selective NMDA glutamate receptor antagonist (* p -value < 0.05, ** p -value < 0.01). Scale bar: 20 μ m.

simple microfabrication tools can be used to spatially isolate the soma of a CNS neuron from its axons and axon-terminals.^{2,3} Using such systems Taylor *et al.* showed that microfluidic channels allow fine manipulation of hippocampal auto-synapses.⁵ In the brain, however, different neuronal populations are connected with well-defined topologies and polarities. Directionality is at the heart of developmental mechanisms involved in the specific connection of different neuronal subtypes. It is also critically involved in the degeneration of neuronal networks in mature brains. Today, no system is able to model a directional network comprising distinct neuronal sub-population so far. In order to obtain directional networks *in vitro*, distinct approaches can be envisioned including axonal mechanical constraints and/or combination of specific gradients of axonal growth cones attractant/repellent. We chose to use physical constraints because their efficiency should not depend on the neuronal type, allowing the use of the same microfluidic system for all neuronal subtypes. The axonal diode concept overcomes the limitation of previous non-polarized systems, allowing a good selectivity towards the allowed connection direction.

The polarity is maintained over time due to several complementary effects (Fig. S1†): first asymmetry imposes directionality by a probabilistic effect, easing the entrance of axons on the wide side of the diode in comparison to the narrowest side. Moreover, we observed that axons from neurons, seeded in the receiving chamber, once encountering the diode wall instead of the micro-channels entrance tend to grow perpendicularly to the diode along the chamber wall, without entering to a great extent the narrow side of the diodes. This effect is probably due to axonal stiffness that prevents frequent axonal turning. Indeed, we noticed that once guided by a parallel microstructure, axons tend to keep that direction for 50 to 100 μ m once exiting the

microstructure. Thus axons encountering a perpendicular wall will turn and keep the new direction. Finally, once emitting axons enter the receiving chamber it is likely that a second filtering effect occurs *via* steric hindrance of the micro-channels that have been filled. Therefore these complementary effects allow a long lasting polarity.

Technically, this system involves a moderate increase in the difficulty of microfabrication; it requires more care and resolution due to the smaller size of the structures, but it remains within the scope of standard microfabrication clean rooms. Indeed, as proposed in the Experimental section, the use of a polymer resin secondary master made from a primary silicon template further increases fabrication simplicity and can be used for years. This way, once a primary master has been prepared, hundreds of microfluidic chips can be prepared without a clean room, directly in the biology lab if necessary.

The directionality of neuronal pathways being a key feature of the nervous system organization, the axon diode concept brings a paradigmatic change in the potential of microfluidic neuron culture platforms for biological studies. Reconstructing a fully mature, functional and oriented neuronal network, in which each cell population and subcellular compartment can be addressed independently, is an important step in bridging the gap currently separating *in vivo* and *in vitro* studies. While *in vivo* studies are indisputably realistic regarding neuronal architectures, they remain very limited for studying molecular and cellular mechanisms. In contrast, *in vitro* studies performed so far allowed molecular and cellular investigations, but lacked a physiologically realistic connectivity. Our microfluidic platform reconciles these two concerns by giving access to precise cellular and molecular dissections in a physiological context.

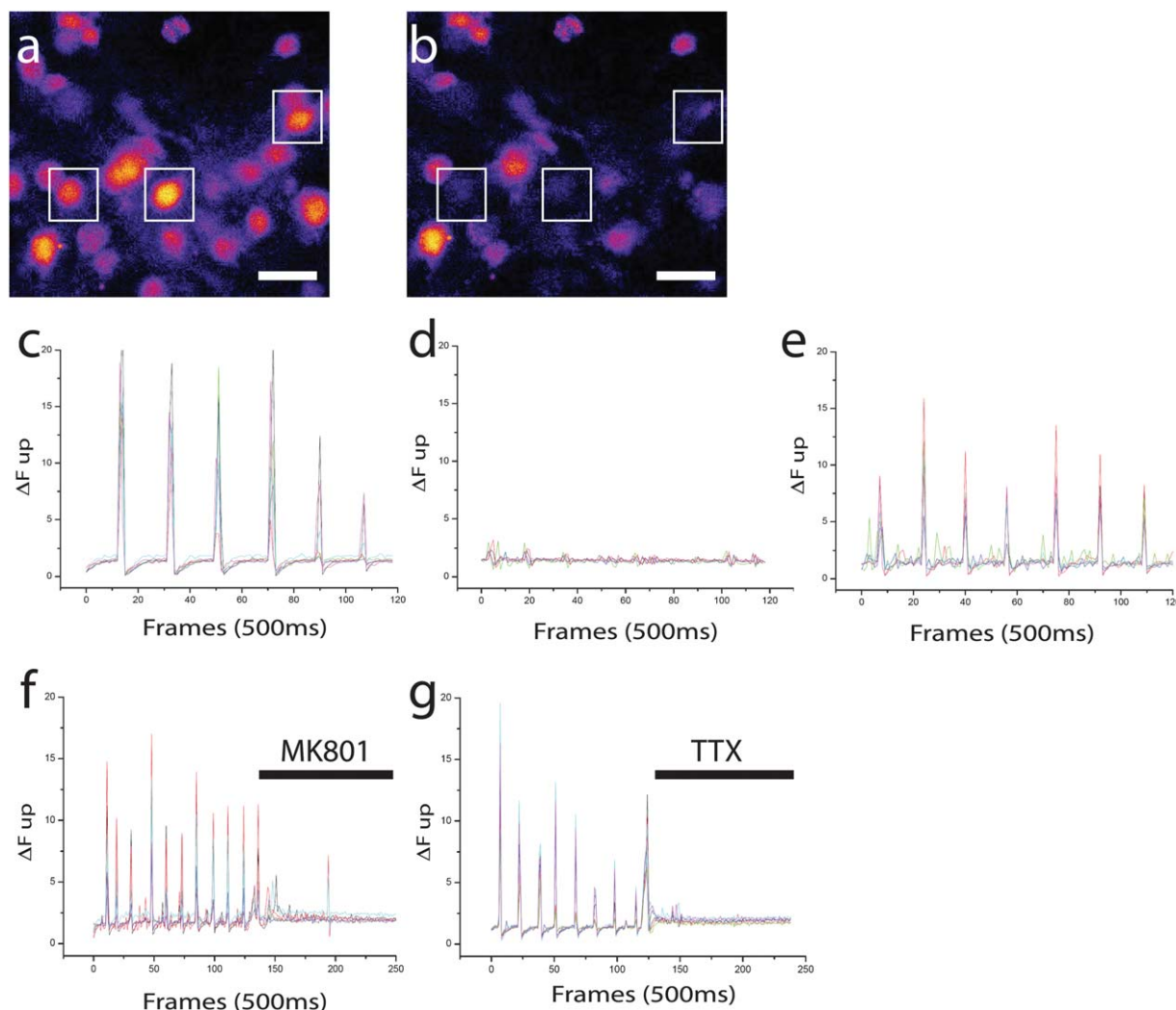


Fig. 6 Striatal neurons acquire slow calcium oscillation. (a and b) Snapshot of Fluo4 oscillation of 15 days old cortical cultures (40 $\times$ magnification, pseudo colors: (a): up, (b): resting). 50% of cortical neurons shows synchronous sign of oscillation. Representative calcium traces analysis of 15 days old, cortical neurons (c), unconnected striatal neurons (d), connected striatal neurons (e). Connected striatal neurons before and after 10 μ M MK801 striatal perfusion, (f), connected striatal neurons before and after 1 μ M TTX striatal perfusion (g). Fluo4 signal oscillations of individual cells from the same experiment were plotted on the same graph.

Reconstruction of a physiologically active network and its importance for neuronal differentiation

As a proof of concept, we reconstructed *in vitro* the cortical-striatal neuronal pathway. The striatum is the principal component of the basal ganglia, which is composed of the caudate, putamen, and accumbens nuclei. Basal ganglia form recurrent circuits critical for motivation and motor planning. The striatum receive glutamatergic inputs from both cortical and thalamic afferents and dopaminergic inputs from the substantia nigra in the midbrain.^{6,7,15} Ninety to 95% of the striatal neurons are GABAergic inhibitory medium spiny neurons (MSN)¹⁶ which have a medium-sized cell body with branched dendrites presenting a high density of dendritic spines. *Via* their projections, striatal neurons give rise themselves to a direct (striato-nigral) and an indirect (striatopallidal) pathway. One of the targets of these two pathways is the thalamus, which in turn sends axons to the cortex leading to a retro-control feedback

loop. The activity of the striatum is mainly driven by excitatory connections coming from diverse areas of cerebral cortex, including sensory, motor and association regions that project to the striatum mainly through ipsilateral glutamatergic projections. This is a purely directional pathway in which the cortical fibers, originating from pyramidal neurons of the layer V, invade the striatum and connect striatal dendrites "en passant",¹⁵ a single cortical axon connecting several striatal neurons. In our system, cortical fiber reconnection on striatal dendrites did trigger both presynaptic clustering along striatal dendrites and striatal neuron maturation. This involves an increase in striatal dendritic arborization complexity, PSD95 clustering and dendritic spine genesis. These observations are in line with previous studies showing that striatal differentiation is partially under the control of cortical neurons.¹⁷ The examination of cortical fibers transduced by a Sindbis viral vector encoding the m-cherry fluorescent protein in cortical cells indicated that cortical axons, which are highly branched in our culture

conditions and grow over more than 2 mm, connect striatal dendrites on their way without stopping, a single cortical axon connecting several striatal neurons (Fig. 2g). This reproduces *in vivo* data on the establishment of “en passant” glutamatergic contacts between cortical axons and striatal neurons.¹⁸ These uni-directional cortico-striatal connections were fully functional: the spontaneous slow calcium oscillation in the cortical population was transferred to the striatal one, whereas in the absence of connection with cortical axons, the striatal population did not show any oscillation. Furthermore, KCl-induced depolarization of cortical neurons reproduced ERK activation in striatal neurons, as observed *in vivo* after electrical stimulation of the cortico-striatal projections.¹⁰ Thus our platform directs neuronal connections, allows separate labeling and stimulation of different cell populations, and allows the observation of various cellular subcompartments at high resolution.

Conclusions and perspectives

In this study we propose the concept of axonal diodes, allowing the *in vitro* reconstruction of oriented and compartmentalized neuronal networks with physiologically active synaptic connections mimicking *in vivo* architectures.

These experiments only represent a very small fraction of the potential applications of the *in vitro* reconstruction of polarized neuronal networks. For instance, more complex networks, involving 3 neuronal populations or more, will be at hand, thanks to a moderate increase in the complexity of the micro-channels array set-up. An obvious application is the investigation of the molecular and cellular mechanisms underlying synaptic and neuronal dysfunction during Alzheimer's, Parkinson's and Huntington's diseases, or prion diseases.^{19–21} Furthermore, this system is also a valuable tool to address developmental issues such as the dynamics of neural networks and synapse formation. Interestingly, too, it will allow the construction of neuron arrays imposing *ad hoc* non-physiological connectivities, providing additional levers to test biological hypotheses regarding *e.g.* neuron maturation and communication. Finally, the axon diodes microfluidic platform could easily integrate microelectrodes, and thereby its range of application could be extended to electrophysiology and cognitive sciences.

Experimental

Chip design and master fabrication

The microfluidic channels comprise two types of component: large channels (50 μm in height) for cell injection and thin channels (2 or 3 μm in height) for axon growth. To fabricate the template with elements of two different depths, we used two layers of photoresist (SU82002 and SY355). A silica wafer was heated to 150 °C and activated by plasma treatment (30 s). A first layer of SU82002 (Microchem) was spin-coated onto the wafer at 2000 rpm and then soft-baked at 65 °C for 2 min and 95 °C for 4 min. The template was then exposed to UV light through an optic mask (made of plastic or quartz if the required resolution was less than 8 μm). After hard bake, the channels (2–3 μm high) were developed in an SU8 developer. The injection channels were then fabricated by laminating the latter wafer with a 55 μm thick layer of dry film SY355 (Elga Europe) at 70 °C. A second mask

was aligned over the SU8 channels and after exposure to UV light, the channels were baked for 5 min at 120 °C and developed in a BMR developer, then rinsed with BMR rinse and isopropanol.

The quality of the narrowing micro-channels was assessed by white-light optical profiling (WLOP) in the vertical scanning mode (VSI), with an interference microscope Wyko® NT1100 (Veeco Instruments Inc., Plainview, NY, USA). The 3D representation was obtained with a 50 $\times$ magnification objective.

Silicon templates were replicated within a polyester resist (Dalbe), which is more robust than silicon wafers. These templates were fabricated by pouring a mixture of polyester base and curing reagent (2%) over a PDMS replica that was previously heated to 110 °C. The resist was cured at 110 °C for 20 min and then at 150 °C for at least 3 h. Two different microfluidic chips were constructed in this way. The first (“2C”) is a two-compartment chip made of two rectangular macro-channels (length: 4000 μm ; width: 500 μm ; height: 55 μm) separated by arrays of asymmetrical micro-channels (length 500 μm , 15 to 3 μm width, 3 μm height; see Fig. 1 a–c).

Microfluidic chip production

PDMS (Sylgard 184) was mixed with curing agent (9 : 1 ratio) and degassed under vacuum. The resulting preparation was poured onto a polyester resin replicate and reticulated at 70 °C for 2 hours. The elastomeric polymer print was detached and 2 reservoirs were punched for each macro-channel. The resulting piece was cleaned with isopropanol and dried. The polymer print and a glass cover slip were treated for 1 minute in an air plasma generator (40% power, 300 mTor, Diener) and bonded together. Chips were placed under UV for 15 minutes and then coated with a solution of poly D-lysine (10 $\mu\text{g ml}^{-1}$) overnight and washed with PBS before cell seeding.

Primary neuronal cultures

Cortices and striata were micro-dissected from E14 embryos of Swiss mice (René Janvier, France) and of a transgenic mice strain expressing GFP (green fluorescent protein) under the control of an actin promoter in all cell types.²² Animal studies were carried out in accordance with the standard ethical guidelines (European Community guidelines on the care and use of laboratory animals: 86/609/EEC). The laboratory animal facility was approved by prefectural decision of the Bureau of Sanitary Police, Paris Prefecture, #DTPP 2010-1230, Nov. 4, 2010. All dissection steps were performed in cold PBS supplemented with 0.1% glucose. Dissected structures were digested with trypsin–EDTA (Gibco) and mechanically dissociated with a pipette. After several rounds of rinsing with phosphate-buffered saline (PBS), cells were re-suspended in DMEM, to a final density of 40 million cells per ml for cortices and 15 million per ml for striata. Cortical cells were then seeded in the somatic compartment and striatal cells in the distal compartment: 3 μl of the cell suspension was introduced into the upper reservoir and cells flowed into the chamber and adhered within 1–2 minutes. The cell culture medium was then added equally to the four reservoirs (40 $\mu\text{l/reservoir}$). Both neuronal cell types were grown in DMEM glutamax + streptomycin/penicillin (Gibco) + 10% FBS + N2 (Gibco) + B27

(Gibco). Microfluidic chips were placed in plastic Petri dishes containing H₂O–EDTA to prevent evaporation and incubated at 37 °C in a humid 5% CO₂ atmosphere. The culture medium was renewed every 3 to 5 days. Upon differentiation, 2 or 3 days after seeding, cortical axons entered the micro-channels and reached the second chambers after 4 to 5 days. Cortical axons continued growing thereafter. Flows were controlled by gravity pressure; each macro-chamber is connected to two reservoirs punched in a reproducible way, thus seeding these reservoirs with specific volume ensure control of flow rate for the desire amount of time.

Immunofluorescence

At various times, depending on the phenomenon under study, cultures were fixed in 4% paraformaldehyde (PFA) for 20 minutes at room temperature. Cells were then washed twice with PBS for 5 min and permeabilized for 45 min with 0.2% Triton X-100 and 1% BSA in PBS. Primary antibodies were then added and the samples incubated at 4 °C overnight in PBS. The samples were rinsed twice for 5 minutes with PBS and further incubated with the corresponding secondary antibodies for 2 hours at room temperature. The chips were then rinsed once with PBS and once with PBS + 0.1% sodium-azide and mounted in a mowiol mounting medium. The following primary antibodies were used: α or β -tubulin-FITC (monoclonal 1/700, sigma); MAP-2 (mouse monoclonal 1/400, Sigma); synaptophysin (mouse monoclonal 1/400, Sigma); VGLUT1 (rabbit polyclonal 1/500, gift E. Herzog, CNRS, UMR 7224); PSD95 (rabbit polyclonal 1/200 cell signaling); GAD67 (mouse monoclonal 1/500, Millipore); Darpp32 (mouse monoclonal 1/500, gift J-A. Girault, Inserm, UMR-S839) and anti-phospho ERK antibodies (mouse monoclonal 1/1000, cell signaling). Species-specific secondary antibodies coupled to Alexa 350, 488, and 555 were used (1/500, Invitrogen) to visualize bound primary antibodies.

Neuronal network stimulation

To decrease serum-induced ERK activation, 13 to 15 day old pure striatal cultures or reconstructed cortico-striatal networks were serum starved for at least 6 hours. To stimulate cortical neurons, the cortical chamber was subjected to DMEM containing 90 mM KCl for 20 minutes. In some experiments, 10 μ M MK801 was introduced into the striatal chambers prior to KCl stimulation. Cell cultures were then fixed with PBS containing 4% PFA and further processed for P-ERK or neuronal immunocytochemistry. To quantify ERK stimulation in striatal neurons, random fields of MAP-2 positive neurons apposed along cortical fibers were counted for phospho-ERK immunoreactivity. Reported values are means from three independent experiments each performed in triplicate.

Image acquisition

Images were acquired with an Axio-observer Z1 (Zeiss) fitted with a cooled CCD camera (CoolsnapHQ2, Roper Scientific). The microscope was controlled with Metamorph software (Molecular Imaging) and images were analyzed using Image J software.

Non-ratiometric calcium analysis

After 12 to 16 days *in vitro*, micro-chambers were rinsed twice with loading buffer (NaCl 129 mM, KCl 4 mM, MgCl₂ 1 mM, CaCl₂ 2 mM, Glucose 10 mM, Hepes 10 mM dissolved in sterile water). Cells were then incubated for 30 min at RT, with 2 μ M fluo4 calcium probe (Invitrogen) dissolved in loading buffer. After 2 rinses cells were returned in DMEM cell culture medium and recorded, at room temperature, under the microscope (see above). Each frame was acquired every 500 ms for 2 to 3 minutes. Calcium trace analysis was performed using Delta F up plugin (WCIF image J plugin) under Image J software. Individual cells corrected fluorescent intensity oscillation were then plotted with Origin 6 software. In some experiments, 10 μ M MK801 or 1 μ M TTX was flown, during acquisition, in the striatal compartment.

Quantification of axonal projections

After fixation and immuno-staining of axonal tubulin (β -tubulin), whole chip images were captured at 400 $\times$ magnification using the stitching function of Metamorph software (Molecular Imaging). Due to the high capacity of cortical axons to form axonal collaterals, the number of axons invading the receiving (or emitting) chambers was estimated by manually counting individual axon exiting each micro-channel at a distance of 50 μ m from the exit of the micro-channel. This gives an estimation of the number of axons entering the chamber independent of their ramification. Selectivity of the diode design was estimated by comparing the number of axons exiting the micro-channels when cortical neurons were seeded at same density on the emitting or the receiving sides 8 days after seeding.

Statistical analysis

For morphological and phosphorylated ERK analyses, differences were assessed by an unpaired Student's *t* test from three independent experiments in which each experimental condition has been performed as a triplicate. For all analysis: * *p*-value < 0.05; ** *p*-value < 0.01; *** *p*-value < 0.001.

Author contributions

JMP invented the diode design, performed the initial neuronal network reconstruction, calcium analysis and wrote the paper. BD performed neuronal network reconstruction, characterization and wrote the paper. LS contributed to the diode design and MV performed the microfabrication experiments. SM and MP performed some cortical cultures. SB initiated the striatal cultures protocol. PVH performed calcium analysis and JC designed the pERK experiment. JLV supervised the microfluidic work and BB supervised the biological work.

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Synapto-Protective Drugs Evaluation in Reconstructed Neuronal Network

Bérangère Deleglise^{1,2}, Benjamin Lassus^{1,2}, Vaneyssa Soubeyre^{1,2}, Aurélie Alleaume-Butaux^{3,4}, Johannes J. Hjorth^{6*}, Maéva Vignes^{1,5}, Benoit Schneider^{3,4}, Bernard Brugg^{1,2}, Jean-Louis Viovy⁵, Jean-Michel Peyrin^{1,2*}

1 Neurobiologie des Processus Adaptatifs, CNRS, UMR7102, Paris, France, **2** Université Pierre et Marie Curie, Paris, France, **3** Inserm UMR-S 747, Paris, France, **4** Université Paris Descartes, Sorbonne Paris Cité, UMR-S 747, Paris, France, **5** Macromolécules et Microsystèmes, CNRS UMR168, Institut Curie, Paris, France, **6** Integrative Neurophysiology, Center for Neurogenomics and Cognitive Research (CNCR), VU University Amsterdam, Amsterdam, The Netherlands

Abstract

Chronic neurodegenerative syndromes such as Alzheimer's and Parkinson's diseases, or acute syndromes such as ischemic stroke or traumatic brain injuries are characterized by early synaptic collapse which precedes axonal and neuronal cell body degeneration and promotes early cognitive impairment in patients. Until now, neuroprotective strategies have failed to impede the progression of neurodegenerative syndromes. Drugs preventing the loss of cell body do not prevent the cognitive decline, probably because they lack synapto-protective effects. The absence of physiologically realistic neuronal network models which can be easily handled has hindered the development of synapto-protective drugs suitable for therapies. Here we describe a new microfluidic platform which makes it possible to study the consequences of axonal trauma of reconstructed oriented mouse neuronal networks. Each neuronal population and sub-compartment can be chemically addressed individually. The somatic, mid axon, presynaptic and postsynaptic effects of local pathological stresses or putative protective molecules can thus be evaluated with the help of this versatile "brain on chip" platform. We show that presynaptic loss is the earliest event observed following axotomy of cortical fibers, before any sign of axonal fragmentation or post-synaptic spine alteration. This platform can be used to screen and evaluate the synapto-protective potential of several drugs. For instance, NAD⁺ and the Rho-kinase inhibitor Y27632 can efficiently prevent synaptic disconnection, whereas the broad-spectrum caspase inhibitor zVAD-fmk and the stilbenoid resveratrol do not prevent presynaptic degeneration. Hence, this platform is a promising tool for fundamental research in the field of developmental and neurodegenerative neurosciences, and also offers the opportunity to set up pharmacological screening of axon-protective and synapto-protective drugs.

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Competing Interests: Patent pending: Dr. JMP, BB and JLV submitted a patent application entitled "DEVICE FOR CELL CULTURE" (CNRS DAE Jun, 15 2011: EP2331673 A2). The authors declare that the "axon diode" microfluidic chip device (Peyrin et al, 2011, Lab Chip) used in the present study is covered by a patent inquiry PCT "Device for cell culture" WO2010040920. National phase patent numbers derived from that PCT inquiry are JP2012504949 (Japan); EP2331673 (EC); CA2739771 (Canada) and US2011306041 (United States). The authors declare no other conflicts of interest. The authors confirm that this does not alter their adherence to the PLOS ONE policies on sharing data and materials.

* E-mail: jean-michel.peyrin@upmc.fr

† Current address: Cambridge Computational Biology Institute, Department of Applied Mathematics and Theoretical Physics, University of Cambridge, Cambridge, United Kingdom

Introduction

In chronic neurodegenerative syndromes such as Alzheimer's and Parkinson's diseases, or acute syndromes such as ischemic stroke or traumatic brain injuries, neuronal degeneration proceeds through a protracted dying-back pattern in which dysfunction of nerve terminals precedes neuronal cell body destruction [1,2,3]. Progressive loss of synaptic connections leads to the lack of controlled synaptic transmission, generation of abnormal neurotransmission, and a reduced level of trophic factors, resulting in impairment of postsynaptic neuronal survival and therefore progressive weakening of the robustness of interconnected networks. These events may account for early cognitive symptoms in patients [4,5]. Synapse failure thus plays a critical role in the

initiation and progression of many neurodegenerative conditions [6,7,8,9]. Moreover, as evidenced by Braak and co-workers, degeneration associated with Alzheimer's or Parkinson's diseases follows specific neuronal pathways in the brain [6,7,8,9,10,11,12]. Together, these observations highlight the need to study neurodegenerative processes at the neuronal network level [13,14,15]. Synaptic degeneration being an early and seminal process in various acute and chronic degenerative syndromes, it is important to develop efficient neuroprotective strategies that target molecular mechanisms involved in synaptic collapse.

The human brain is a complex organ composed of (~200) billion cells and even more complex interconnected cellular interactions. Current experimental models used to study synaptic

stability under pathological conditions range from whole animal models that preserve the anatomical structures but greatly limit experimentation at cell level, to dissociated cell culture systems that allow detailed manipulation of cell phenotypes but lack the highly ordered and instructive brain environment. In previous work we and others demonstrated that microfluidic technologies for cell culture are powerful tools allowing exquisite control of the neuronal micro-environment and offering the possibility to reconstruct fully functional neuronal pathways *in vitro* [16,17,18,19], for review see [20,21], thus paving the way to study neurodegenerative syndromes on chips. In the present study we present a microfluidic platform allowing axotomy of reconstructed neuronal pathways and show that chemical axotomy of cortical fibers triggers a rapid presynaptic disconnection from striatal dendrites. Our synaptic damage assay recapitulates the molecular events previously mapped during *in vivo* experiments. We perfused pharmacological compounds targeting specific molecular pathways in the synaptic compartment, and used automated image analysis quantification of presynaptic collapse. We demonstrate that a rho Kinase inhibitor (Y27632), and nicotinamide adenine dinucleotide (NAD⁺), exert strong synapto-protective activities whereas zVAD-fmk and resveratrol fail to protect synapses. We propose the use of this sophisticated “brain on chip” as a versatile platform for fast evaluation of synapto-protective drugs.

Material and Methods

Chip Design and Master Production

Microfluidic channels are made up of two elements: 55 μm high macro-chambers for cell or fluid injection, separated by narrowing arrays of 3 μm high micro-channels allowing directional axonal outgrowth. The three compartmented chip (“3C”) was constructed using the following design: two rectangular macro-channels (length 4000 μm , width 500 μm , height 55 μm) separated by arrays of asymmetrical micro-channels (length 500 μm , width 15 to 3 μm , height 3 μm , [17]) interrupted by a third macro-channel inserted in the middle of the micro-channel “diode” array (see Fig. 1a,b). This third macro-channel acts as an intermediate compartment allowing control of the flow of solution over the mid-portion of cortical axons [16].

To produce the template with elements of two different thicknesses, we used two layers of photoresist (SU82005 and SY355). A silica wafer was dehydrated by heating it to 150°C for 10 minutes and activated by plasma treatment (30s). A first layer of SU82005 (Microchem) was spin-coated onto the wafer at 3800 rpm and then soft-baked at 65°C for 2 minutes and 95°C for 4 minutes. The template was then exposed to UV light through an optic mask (made of plastic or quartz if the required resolution was less than 8 μm). After hard bake, the channels (3–4 μm high) were developed in SU8 developer. The injection channels were then produced by laminating the newly obtained wafer with a 55 μm -thick layer of dry film SY355 (Elga Europe) at 90°C. A second mask was aligned over the SU8 channels and after exposure to UV light, the channels were baked for 10 minutes at 120°C and developed in BMR developer, then rinsed with BMR rinse, acetone and isopropanol.

The quality of the narrowing micro-channels was assessed by white-light optical profiling (WLOP) in vertical scanning mode (VSI), with an interference microscope Wyko® NT1100 (Veeco Instruments Inc., Plainview, NY, USA); this is a non-contact optical profiling system, based on light interferences, that provides high vertical resolution.

Microfluidic Chip Production

Polydimethylsiloxane (Sylgard 184, PDMS, Dow Corning, Midland, MI, USA) was mixed with a curing agent (9:1 ratio) and degassed under vacuum. The resulting preparation was poured onto a polyester resin replicate and reticulated at 70°C for 2 hours. The elastomeric polymer print was detached and two reservoirs were punched for each macro-channel. The resulting piece was cleaned with isopropanol and dried. The polymer print and a glass cover slip were treated for 200 seconds in an air plasma generator (98% power, 0.6mBar, Diener Electronic, Ebhausen, Germany) and bonded together. The chips were placed under UV for 15 minutes and then coated with a solution of poly-D-lysine (10 $\mu\text{g}/\text{mL}$, Sigma; St. Louis, MO, USA) overnight and washed with PBS before cell seeding.

Primary Neuronal Cultures

Animal care was conducted in accordance with the standard ethical guidelines of the CNRS “Formation à l’Expérimentation Animale” and approved by the “C2EA - 05 Comité d’éthique en expérimentation animale Charles Darwin”. Timed pregnant mice at E14 were purchased from René Janvier and cared for by the well-established animal care facility at University Pierre et Marie Curie (IFR83). Pregnant mice were decapitated and cortices and striata were micro-dissected from E14 embryos of Swiss mice (Janvier, Le Genest Saint Isle, France) and of a transgenic mice strain expressing GFP (green fluorescent protein) under the control of an actin promoter in all cell types [22]. All steps of dissection were in cold phosphate buffer saline (PBS) supplemented with 0.1% glucose (Life Technologies, Inc., Gaithersburg, MD, USA). Dissected structures were digested with trypsin-EDTA for striata (Life Technologies, Inc., Gaithersburg, MD, USA) or papain for cortices (20U/ML in DMEM, Sigma; St. Louis, MO, USA). After trypsin or papain inactivation with fetal bovine serum (FBS, PAA Piscataway, NJ, USA), structures were mechanically dissociated with a pipette in presence of DNase. After several rounds of rinsing, cells were re-suspended in DMEM (Life Technologies, Inc., Gaithersburg, MD, USA), to a final density of 45 million cells/ml for cortices and 12 million cells/mL for striata. Cortical cells were then seeded in the somatic compartment and striatal cells in the distal compartment: 3 μL of the cell suspension was introduced into the upper reservoir and cells flowed into the chamber and adhered within 1–2 minutes. Cell culture medium was then added equally to the four reservoirs (60 $\mu\text{L}/\text{reservoir}$). Both neuronal cell types were grown in DMEM glutamax+streptomycin/penicillin (Life Technologies, Inc., Gaithersburg, MD, USA) +10% FBS+N2+ B27 (Life Technologies, Inc., Gaithersburg, MD, USA). Microfluidic chips were placed in plastic Petri dishes containing H₂O-EDTA to prevent evaporation and incubated at 37°C in a humid 5% CO₂ atmosphere. The culture medium was renewed every six days. Upon differentiation, 2 or 3 days after seeding, cortical axons entered the micro-channels and reached the second chambers after 5 to 6 days. Cortical axons continued growing thereafter.

Pharmacological Treatment

All chemicals were prepared as concentrated solution according to the recommendations of the different manufacturers. Compounds were aliquoted in Eppendorf tubes and used once to avoid repeated freezing/thawing processes. Aliquots were stored at –80°C for no longer than two months. Care was taken to protect photosensitive molecules from light by wrapping the test tubes in aluminum foil. Drugs were extemporaneously diluted at their respective final concentration in DMEM containing 10% SVF+N2+ B27.

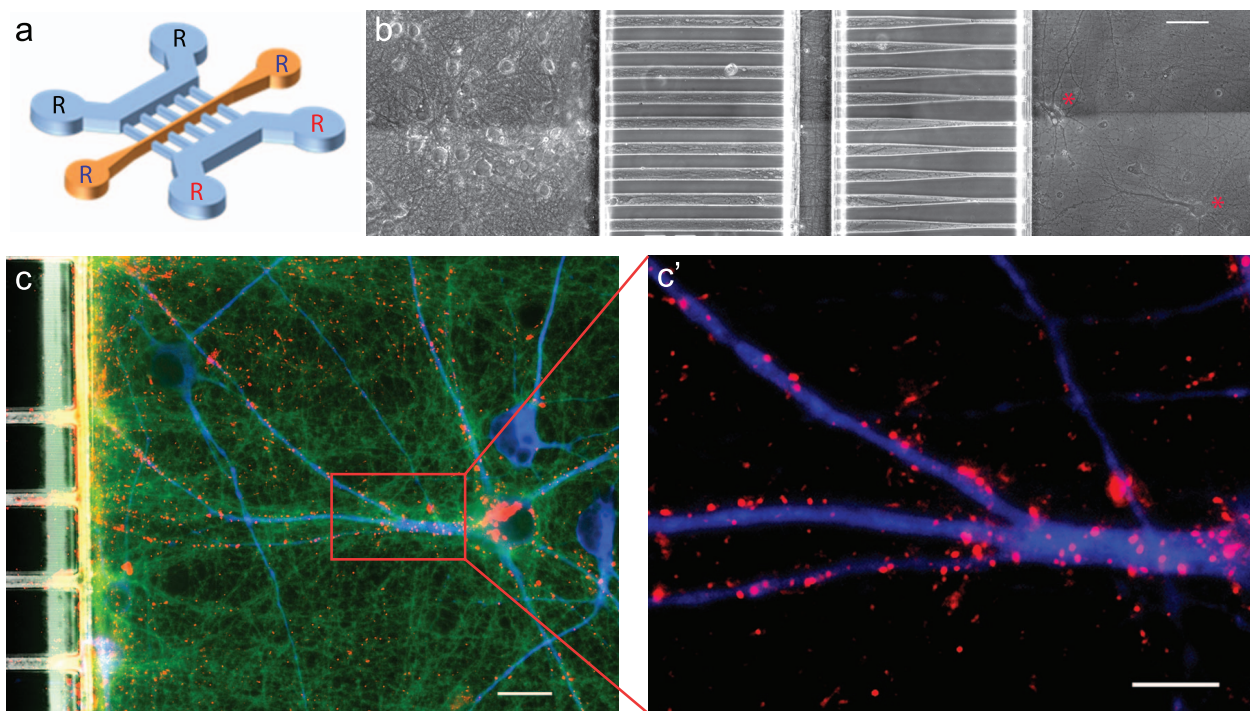


Figure 1. Reconstruction of oriented neuronal networks in 3C-microfluidic chip. **a:** Microfluidic neuronal culture devices are made up of two separate cell culture chambers (blue) interconnected by a series of asymmetrical micro-channels interrupted by a central narrow (50 μm -wide) channel that gives access to the central part of the axons. Each chamber is perfused individually by two reservoirs (indicated by R). **b:** Phase contrast image of a reconstructed oriented neuronal network in 3C-chip. Cortical neurons are seeded in the left chamber and connect striatal neurons seeded in the right chamber (red stars). The size of asymmetrical micro-channels allows the outgrowth of axons but prevents the entry of cell bodies; micro-channels allow the passage of axons only in a left-to-right direction (axonal diodes) *scale bar* = 50 μm . **c–c':** Immunofluorescent images of the receiving (right) chamber. Cortical axons exit micro-channels (green: α -tubulin) and cluster presynaptic markers (red: v-GLUT1) on striatal dendrites (blue: MAP-2). **c':** Enlargement of the clustering of cortical presynaptic labeling on striatal dendrites, suggesting cortico-striatal synapse establishment. *scale bar* = 20 μm .
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For pharmacological pretreatments, medium was removed from the striatal chambers and was replaced by DMEM containing the pharmacological drugs. Except for NAD^+ , which was administered overnight, pharmacological compounds were perfused one hour before axotomy. The following concentrations were used for control or axotomy conditions: resveratrol (20 μM , Sigma; St. Louis, MO, USA), z-VAD (50 μM , Sigma; St. Louis, MO, USA), Y27632 (10 μM , Sigma; St. Louis, MO, USA) or NAD^+ (5 mM, Sigma; St. Louis, MO, USA). After axotomy of the central channel, fresh medium containing drugs was applied to wash detergent.

Live Imaging

Live imaging of striatal dendritic spines was carried out using a transgenic mouse expressing GFP under the control of an actin promoter [22]. Non-transgenic cortical neurons were seeded in the emitting chamber, while striatal neurons from the GFP-expressing mouse were cultivated in the receiving chamber. Fluorescent live imaging of striatal dendritic spines was carried out in control or axotomized conditions for 6 hours using a 63X oil-objective.

Immunofluorescence

At various times, cultures were fixed in 4% paraformaldehyde (PFA, Sigma; St. Louis, MO, USA) for 20 minutes at room temperature. Cells were then washed twice with PBS for 5 minutes and permeabilized for 45 minutes with 0.2% Triton X-100 and 1% BSA in PBS. Primary antibodies were then added and the

samples incubated at 4°C overnight in PBS. The samples were rinsed twice for five minutes with PBS and further incubated with the corresponding secondary antibodies for two hours at room temperature. The chips were then rinsed once with PBS and once with PBS+0.1% sodium-azide, and mounted in moviol mounting medium. The following primary antibodies from Sigma (Sigma; St. Louis, MO, USA) were used: α -tubulin-FITC (monoclonal 1/700); Microtubule Associated Protein -2 (MAP-2, mouse monoclonal 1/500); Synaptophysin (mouse monoclonal 1/500); vesicular glutamate transporter 1 (v-GLUT1, rabbit polyclonal 1/500, gift E. Herzog, CNRS, UMR 7224). Species-specific secondary antibodies coupled to Alexa 350, 488, or 555 were used (1/500, Life Technologies, Inc., Gaithersburg, MD, USA) to visualize bound primary antibodies.

Neuronal Network Axotomy

After full maturation of the neuronal network (14 days after seeding), cell culture medium was removed from the two reservoirs connecting the central chamber, and solution of plain DMEM (sham) or 0.1% Triton in DMEM was flowed from the upper reservoir for 30 seconds. Careful attention was paid to the fluidic isolation of the central channel: the two outer cell culture chambers were pressurized with 50 μl of cell culture medium while 20 μl of sham or detergent-containing medium were introduced into the upper reservoir of the central channel. This created a flux of detergent into the central chamber containing the cortical axons. The central chamber was then washed three times

with DMEM and the central reservoirs were then filled with medium. Axotomized cells were then kept at 37°C under 5% CO₂ for various periods before fixation.

Image Acquisition

Images were acquired with an Axio-observer Z1 (Zeiss, Germany) fitted with a cooled CCD camera (CoolSnapHQ2, Roper Scientific). The microscope was controlled with MetaMorph software and images were analyzed using ImageJ and SynD software [23].

Quantification of Synaptic Disconnection

Synaptic disconnection was assessed by counting v-GLUT1 clusters affixed to MAP-2 positive striatal dendrites. All images were obtained using the same acquisition parameters. The images were similarly processed with ImageJ software before being used for quantification: the brightness/contrast of all control images was optimized manually to eliminate the background and to maximize the signal. The means of the minimum and maximum intensities were then calculated in the control condition and these settings were applied to all images. The images of the three stainings v-GLUT1/ α -Tubulin/MAP-2 were merged and the resulting image was used to define the zone where striatal dendrites were sufficiently innervated by cortical fibers. A v-GLUT1/MAP-2 merge was then used for quantification using SynD software (Fig. S1). The merged v-GLUT1/MAP-2 images (Fig. S1A) were analyzed using SynD [23]. Here the somata were detected by thresholding the MAP-2 channel, and smaller structures such as neurites were removed by morphological erosion followed by dilation (Fig. S1B). Neurites were detected using steerable filters applied to the MAP-2 channel (Fig. S1C). Finally, synapses were detected by thresholding the v-GLUT1 channel to get a synapse mask (Fig. S1D) and then putative individual synapse centers were detected by deconvolving the original v-GLUT1 channel and locating peaks (Fig. S1E). Only synapses detected on or near the neurites were included for analysis. The resulting synapse counts were then exported to Excel for further analysis. The settings used were: Soma erode = 25; Morphology threshold = 35; Max cost = 0.9; Steerable filter size (in μ m) = 1.6; Synapse intensity threshold in standard deviations above noise = 0.3; Neurite padding (zone in μ m outside neurite mask where synapses are counted) = 8; Minimal size = 0.3 μ m. Reported values are means for at least three independent experiments, each performed in triplicate.

Statistical Analysis

For synapse quantification, differences were assessed by ANOVA, followed, when appropriate, by a post-hoc Bonferroni test. For all analyses: *p-value < 0.05; **p-value < 0.01; ***p-value < 0.001.

Results

Setting up a Microfluidic Cell System to Model Axonal Injury in Oriented Neuronal Networks

To assess the impact of axonal injury on distant presynaptic dynamics we designed a microfluidic platform allowing the manipulation of reconstructed neuronal networks encompassing three separate sub-compartments (Fig. 1). This was achieved by combining two approaches previously developed in our laboratories. The first system was designed to allow easy axotomy of neuronal axons in a three-compartment chip presenting a somato-dendritic compartment, a narrow central channel giving access to the central part of the axons, and a distal receiving chamber

containing axons [16]. The second system comprised asymmetrical micro-channels to polarize axonal growth and reconstruct oriented neuronal networks *in vitro* in a two-compartment chip [17].

The new device is composed of a three-compartment chip in which the diode array is interrupted by a 50 μ m-wide central channel (Fig. 1a and b). The system is made up of two distinct cell culture chambers (for each neuron type), each connected to two reservoirs (indicated by an R in Fig. 1a) and separated by an array of 500 μ m-long narrowing micro-channels allowing unidirectional axonal growth (from the proximal chamber to the distal one) and interrupted by a third central channel giving access to the central part of the axons (orange channel in Fig. 1a). Using this multi-compartment device, we next reconstructed an oriented cortico-striatal neuronal network in which the somatic, axonal and synaptic compartments of cortical neurons are specifically accessible through fluidic perfusion. To that purpose, mouse primary cortical neurons were seeded in the left chamber (on the wider side of the narrowing micro-channels), and striatal neurons from the same mouse were seeded in the receiving chamber. Fig. 1b shows phase contrast micrographs of cortical neurons projecting towards striatal neurons. Neurons seeded in the chamber with large micro-channel openings emit axons that invade the micro-channels and grow within these tubes. When exiting the first array of micro-channels, about 80% of the cortical fibers crossed the central chamber, reached the second array of narrowing micro-channels and continued to grow to connect striatal neurons seeded in the receiving cell culture chamber (Fig. 1b). As described previously [17], due to the small size of the micro-channel tips, axons from the receiving striatal chamber bumped into the sidewall of the chamber and did not shoot towards the cortical chamber. Fifteen days after cell seeding, the neuronal network was fully established and cortical fibers were connected to fully differentiated striatal neurons, as evidenced by the docking of VGLUT1 positive presynaptic cortical terminals to MAP-2 positive striatal dendritic arborization (Fig. 1c') and the presence of GFP positive dendritic spines on striatal dendritic shafts. Connected striatal neurons showed slow spontaneous calcium oscillation imposed by cortical spontaneous rhythms (data not shown and [17]). Overall, introducing a central chamber dedicated to the manipulation of the mid portion of cortical fibers did not modify neuronal differentiation in reconstructed networks.

Kinetics of Structural Alterations after Cortical Fiber Axotomy

Synaptic and axonal degeneration are two of the earliest structural alterations occurring in various acute and chronic neuronal pathologies, and often precede the destruction of the cell body. Severing of peripheral or central nerves is widely used as an experimental model of brain and axonal trauma in order to decipher specific molecular pathways involved in axonal degeneration. However, the molecular mechanisms and kinetics involved in presynaptic breakdown of nerve terminals remain elusive. In order to assess the impact of axonal trauma on synaptic terminals we axotomized the reconstructed cortico-striatal neuronal network. Axotomy was carried out by perfusing cell culture medium containing 0.1% Triton for 30 seconds in the central compartment containing cortical axons (Fig. 2a–f). As shown in Fig. 2e we monitored minute severing of cortical axons within the central channels without damaging the proximal parts of the fibers within the micro-channels. We next performed a kinetic analysis of neuronal network component degeneration. The cortical somato-dendritic compartment showed no signs of either tubulin architectural alterations or dendritic alteration following axotomy (MAP-2 and α -tubulin staining, Fig. 2a,d). The portion of axon still

connected to the cortical soma was able to re-grow after axotomy (data not shown), thus indicating that axotomy of cortical fibers does not trigger a retrograde death signaling process. Compared to sham perfused fibers (Fig. 2g), severed cortical axons in the striatal chamber (Fig. 2h) displayed no signs of axonal tubulin fragmentation even two hours after axotomy. Axonal degeneration started four hours after lesion (i) with sparse tubulin beading (axonal blebs); six hours after axotomy all axons were fragmented (j). Meanwhile, the global architecture of the striatal dendritic tree (revealed by MAP-2 labeling) remained globally unaffected during the first six hours.

The number of cortical v-GLUT1 clusters on striatal dendrites was counted by widefield epifluorescence microscopy at various times after axotomy. Under control conditions (Fig. 3a,c), there were approximately 40 v-GLUT1 presynaptic terminals per 100 μm of striatal dendrite. Two hours after axotomy, the number of presynaptic cortical clusters on striatal fibers was 60% lower (Fig. 3d) than in control cultures (Fig. 3c), whereas cortical axons showed no signs of blebbing or disorganization of their microtubular network. Four hours after axotomy (when the first sign of axonal alteration appeared), presynaptic density was 75% lower in axotomized conditions (Fig. 3e) compared to sham experiments (Fig. 3a,c). Finally, six hours after axotomy, when the cortical axons were almost all fragmented (Fig. 2j), presynaptic v-GLUT1 staining had completely disappeared (Fig. 3f), indicating complete destruction of cortico-striatal synapses. We concluded that cortical axotomy triggers a progressive cortico-striatal synaptic disconnection, which precedes the fragmentation of cortical axons.

As shown in Fig. 2g–j, the global architecture of striatal neurons did not seem to be altered during the axotomy time course. However, we wondered whether presynaptic cortical degeneration would induce a milder postsynaptic alteration. Non-transgenic cortical neurons were seeded in the emitting chamber, while striatal neurons from a transgenic mouse expressing GFP (green fluorescent protein) under the control of an actin promoter were grown in the receiving chamber. Fluorescent live imaging of striatal dendritic spines was carried out in control or axotomized conditions for six hours (Fig. 3g–n). In control conditions, striatal spines were stable (Fig. 3g–j). Following cortical fiber axotomy the

striatal spines remained stable even when cortical pre-synapses previously connecting those spines had almost all degenerated (Fig. 3k–n). These observations suggest that presynaptic disorganization represents one of the primary events induced by axonal damage, which in turn induces the anterograde propagation of a fast degenerative signal within the axons.

The loss of presynaptic clusters was also associated with the apparent dispersal of the residual v-GLUT1 vesicles (not shown). There are several possible explanations for this observation. First, v-GLUT1-positive vesicles may disperse along axon fibers due to local modification of the mechanical tension of axonal fibers triggered by axotomy. Second, dispersion of v-GLUT1 and presynaptic proteins may be associated with the collapse of the whole structure of the spine. It would be of interest to monitor the dynamics of v-GLUT1 movement, using v-GLUT1-GFP chimeric protein, to address this issue.

Live imaging of striatal dendritic spines revealed that striatal spines did not degenerate within the same time-window as pre-synapses (compare Fig. 3g–n with Fig. 3a–f). Even four hours after axotomy, when almost all the synapses had degenerated and axons started to fragment, striatal postsynaptic architecture remained intact (Fig. 3m). This suggests that structural postsynaptic integrity can be preserved for at least four hours after the loss of presynaptic structures.

Pharmacological Evaluation of Synapto-protective Drugs in 3C-microfluidic Chip

Using the above data it was possible to identify a time-window within which pre-synapses had almost all degenerated after cortical axotomy while axons or postsynaptic elements remained intact. This time-window can be used to screen for drugs expected to have the capacity to slow down or prevent synaptic loss.

With the help of our microfluidic-based synaptic stability/degeneration assay, we first quantified synaptic degeneration using SynD software (see [23] and Fig. S1), which allows automatic segmentation of striatal dendritic trees and co-localization of v-GLUT1 positive synapses docked to the dendrites. Automatic counting was used to evaluate the number of v-GLUT1 clusters on

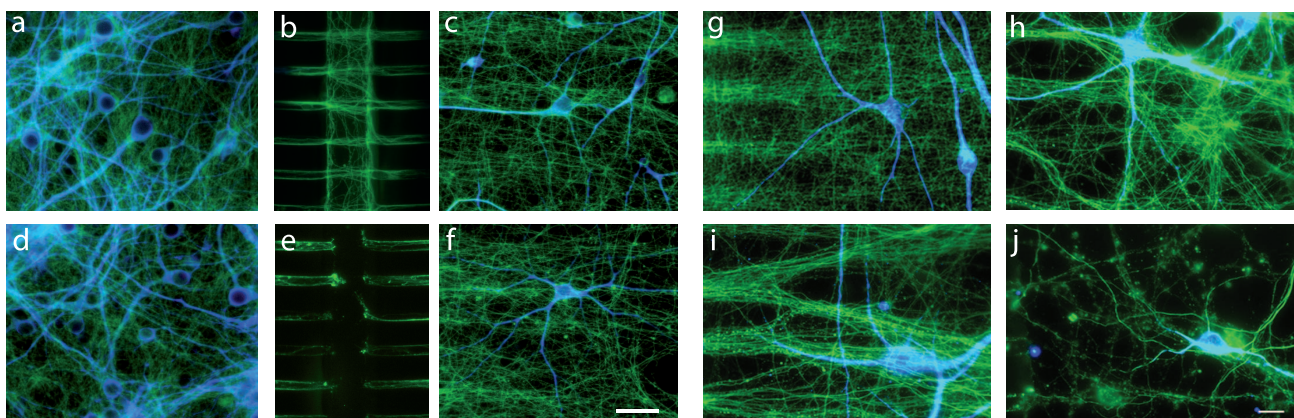


Figure 2. Kinetics of axonal degeneration following cortical axotomy in a network. a,d: Cortical, b,e: central and c,f: striatal, chambers of normal (a–c) or axotomized (d–f) reconstructed cortico-striatal networks (div14). Neurons were stained for MAP-2 (blue), α -tubulin (green) and v-GLUT1 (red). a–c: Cortical neurons send axons efficiently through the micro-channels and the central chamber to reach, and connect with, striatal neurons. d–f: Two hours after axotomy, no axons are present in the central chamber but axons inside the micro-channels and in the striatal chamber remain intact. scale bar = 75 μm g–j: Kinetics of degeneration of the distal part of the cortical axons following axotomy of cortical fibers in the receiving chamber. No axonal fragmentation (assessed by α -tubulin, green) is observed either in non-axotomized condition (g), or two hours after axotomy (h). The first tubulin beads (blebs) on cortical axons appear four hours after axotomy (i), and after six hours, all cortical axons disconnected from their soma are fragmented whereas striatal morphology remains intact (MAP-2, blue) (j). scale bar = 20 μm . doi:10.1371/journal.pone.0071103.g002

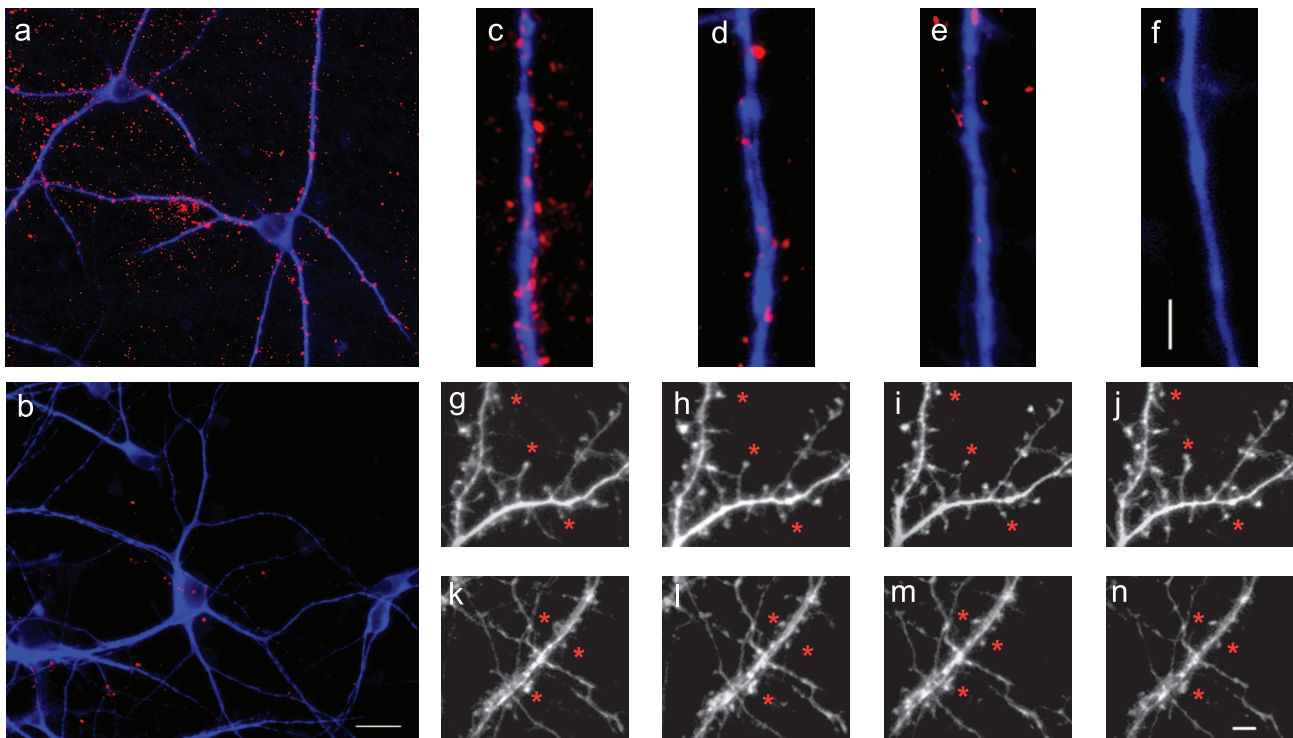


Figure 3. Early cortico-striatal synaptic disconnection after cortical fiber axotomy. **a–f:** Cortical presynaptic structures (v-GLUT1, red) affixed to striatal dendrites (MAP-2, blue) in control conditions (**a**), three hours after axotomy (**b**) scale bar: 20 μ m. Note the disappearance of v-GLUT1 labeling suggesting cortical presynaptic degeneration. **c–f:** Representative high magnification images in control conditions (**c**) and two hours (**d**), four hours (**e**) and six hours (**f**) after axotomy. Note the fast cortico-striatal disconnection as soon as two hours after axotomy. scale bar: 5 μ m. **g–n:** Live imaging of GFP-expressing striatal neurons at time 0h (**g,k**), 2h (**h,l**), 4h (**i,m**) and 6h (**j,n**) in control (**g–j**) or axotomy (**k–n**) conditions. Note the stability of striatal dendritic spines (arrow) in both conditions. scale bar: 15 μ m.
doi:10.1371/journal.pone.0071103.g003

striatal dendrites in control conditions (Fig. 4a–e) or three hours after axotomy (Fig. 4f–j), when cortical pre-synapses have normally degenerated (but not cortical axons or striatal spines).

We then selected several pharmacological compounds known to impede synaptic and/or axonal degeneration *in vivo*. Although the precise molecular events triggered in synaptic destruction upon neurodegenerative stimuli are still unknown, previous experiments conducted after *in vivo* axotomy of peripheral nerves have shown that presynaptic collapse at the neuromuscular junction is triggered by a caspase-independent, NAD^+ -dependent signaling pathway. However, whereas there is no doubt about the involvement of caspases in cell body destruction, their role in axonal and synaptic degeneration is still an open question.

We therefore decided to evaluate the synapto-protective potential of both NAD^+ and z-VAD-fmk, a broad-spectrum caspase inhibitor. The cultures were pretreated for one hour with z-VAD-fmk (50 μ M) (Fig. 4c,h) or for 24 hours with NAD^+ (5 mM) (Fig. 4e,j). Cultures pretreated with NAD^+ and subjected to axotomy retained 65% of the initial number of synapses whereas non-pretreated cultures retained 25% (Fig. 4k). In contrast, no significant synapto-protection was found in cultures pretreated with z-VAD-fmk (Fig. 4k). We also investigated the protective potential of resveratrol on synapses. As resveratrol, an activator of Sirtuins (a group of NAD^+ dependent histone deacetylases) has been shown to have a similar protective effect on axons to NAD^+ (Araki et al, 2004), we decided to evaluate its protective potential on synapses. However, in our experimental conditions, pretreatment with 20 μ M resveratrol (1 h and 24 h) did not show any protective potential on synapses (Fig. 4b,g,k).

Finally, actin being a key component of pre and post-synapses which controls different aspects of synaptic physiology, we included a Rho Kinase inhibitor (Y27632) [24]. Interestingly, a strong synapto-protection was obtained with the Rho-kinase inhibitor (10 μ M, 1 h pretreatment) with 75% of synapses preserved, indicating that ROCK may play an important role in presynaptic stability under stress. Our experimental setup thus successfully demonstrated that cortical axotomy induces synaptic disconnections preceding axonal degeneration and that NAD^+ and Rho-kinase inhibitor have a specific protective effect against this cortico-striatal disconnection.

Discussion

This tri-Compartment (3C)-microfluidic system for neural network reconstruction and pathological process modeling is a good example of the interest of microfluidic for neurosciences. What neuroscientists need are sophisticated but easy to handle biological models to help solve complex neuro-scientific issues [20,21]. Due to the inherent complexity of the organization of the brain, many questions regarding developmental issues, neuronal activities in complex networks and the mechanisms of neurodegeneration in either acute or neurodegenerative diseases remain unanswered. The lack of relevant but simplified models of neuronal networks has slowed down the development of efficient drug therapies which protect synapses and prevent network dysfunctions. Here we show that this new structured network integrating different neuron types that reproduce essential properties of neuronal communication is easy to handle and

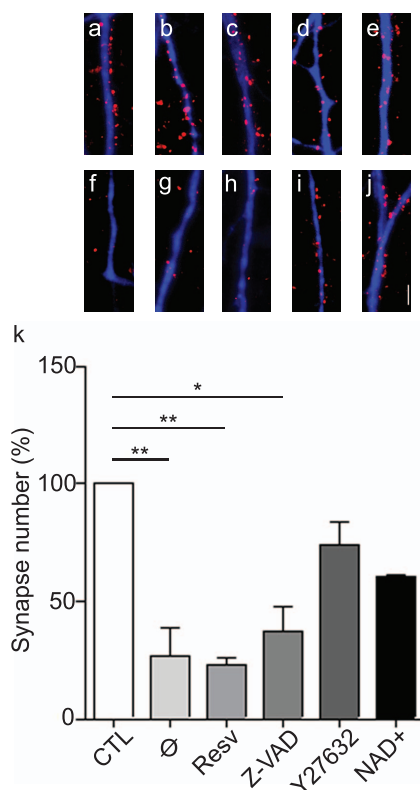


Figure 4. Pharmacological screen of synapto-protective drugs in 3C-microfluidic chip. **a–j:** Representative high magnification images of cortical presynaptic structures (v-GLUT1, red) affixed to striatal dendrites (MAP-2, blue) in non-axotomized (**a–e**) and 3 hours post-axotomy (**f–j**) conditions, without treatment (**a,f**), or with resveratrol (20 μ M **b,g**), 50 μ M z-VAD (**c,h**), 10 μ M Y27632 (**d,i**) or 5 mM NAD⁺ (**e,j**) pre-treatment. *scale bar: 5 μ m.* **k:** Quantification of cortical presynaptic structures affixed to MAP-2-positive striatal dendrites after cortical axotomy. The graph represents the relative number of synapses remaining compared to non-axotomized condition. Y27632 and NAD⁺ delay cortical synaptic loss whereas no significant protection is observed with resveratrol or z-VAD pre-treatment. (ANOVA 2, *p-value<0.05, **p-value<0.01). Synaptic degeneration was quantified using SynD software (see Figure S1) which allows automatic segmentation of striatal dendritic trees and co-localization of VGLUT1 positive synapses docked to the dendrites [23]. doi:10.1371/journal.pone.0071103.g004

greatly facilitates the evaluation of synapto-protective drug potency.

Apoptosis is generally accepted to be a key event triggering neuron death in late-onset neurodegenerative diseases, yet their role in axonal and synaptic degeneration is unclear. Although caspases 3 and 9, which are key effectors of the apoptotic pathway, can be activated locally in synaptic terminals and neurites, resulting in local functional and morphological modifications [25,26], they do not seem to play an active role in axotomy-induced axonal degeneration [27,28]; see review [29,30,31]. Although it has been postulated that modest synaptic caspase activation might be involved in local non-apoptotic mechanisms such as long term depression [25,26], our findings showing that caspase inhibitors failed to protect synapses after acute axonal injury further suggest that caspase activation is not a crucial mechanism in the synaptic degeneration process. Our findings are in line with the data showing that caspases are not involved in Wallerian degeneration processes, where it is rather NAD⁺

signaling which is involved. Indeed, the WldS gene was shown to confer a strong axo and synapto-protective effect at the neuromuscular junction through an age-dependent process. This gene mediates its effects partly through its nicotinamide mononucleotide adenyltransferase-1 NMNAT1 moiety, an NAD⁺ producing enzyme [32]. Interestingly, in the CNS, the WldS gene delays nerve terminal degeneration in the striatum following ablation of the ipsilateral cerebral cortex [33]. Here we show that exogenous NAD⁺ strongly protects synapses after axonal trauma and confirm the importance of NAD⁺ signaling pathways in synaptic maintenance. However, both the targets of NAD⁺ and its site of action remain elusive. NAD⁺ is a molecule involved in many reactions and cellular functions including energetic metabolism, mitochondrial functions, and antioxidant defenses. It has long been postulated that NAD⁺ exerts axo-protective effects through nuclear signaling [34] but recent experiments have demonstrated that extra-nuclear NAD⁺ producing enzymes have the same effect [35]. Thus, although the mechanisms of NAD⁺ synapto-protective effects remain enigmatic, our data suggest that synaptic application of NAD⁺ may act locally to protect synapses after acute axonal injury. Finally, the results we obtained with caspase inhibitors and exogenous NAD⁺ are in line with data gathered from *in vivo* models, and therefore ensure the reliability of our evaluation assay. This encouraged us to assess the synapto-protective properties of two compounds targeting pathways potentially involved in synaptic collapse.

Among NAD⁺ potential targets, sirtuins represent attractive candidates. They are NAD⁺ dependent class III histone deacetylase involved in controlling longevity [36]. Sirtuin 1 (SIRT1) has previously been shown to mediate the axo-protective effect of WldS protein on axotomy [34]. Resveratrol that activates several sirtuins through indirect signaling pathways involving both AMPK activation and NAD⁺ production [37,38], has been shown to have a similar axo-protective effect, once applied to entire neurons [34]. However, these results were not confirmed in another degenerative paradigm [39], and its effect on synapse is not known. It was therefore tempting to assess whether or not local (synaptic and axonal) application of resveratrol could delay synaptic destruction in our paradigm. The failure of synaptic application of resveratrol to directly protect synapses contrasts with the NAD⁺ protective effect. This may be due to the fact that resveratrol targets (either direct or indirect) are not expressed or enriched enough in presynaptic terminals to trigger a strong protective effect. This does not, however, rule out the possibility that resveratrol may exert a synapto-protective effect through long distance signaling such as nuclear SIRT1 activation, for example.

Finally, we wanted to see whether molecules which interfere with actin dynamic could slow down presynaptic collapse triggered by cortical fiber axotomy. In previous work we and others have shown that axotomy of cortical axons triggers a rapid collapse of actin filopodia in unconnected axons [16,40,41]. Pre and post synaptic elements are greatly enriched in actin networks and are virtually devoid of tubulin cytoskeleton. Actin dynamic controls most of the synaptic physiology. Among the effectors that control actin polymerization/depolymerization in neurons, Rho GTPase and Rho Kinase (ROCK), a group of serine/threonine kinases, are important players [42]. While the role of Rho GTPase in controlling dendritic and synaptic dynamic is becoming clearer, little is known about the role of Rho Kinase in synapses. ROCK signaling was recently involved in mediating growth cone collapse triggered by chemo-repulsive cues during brain development [43] and in controlling the readily releasable pool of synaptic vesicles [44]. Our results demonstrating that Y27632 is a potent inhibitor of presynaptic collapse show that ROCK are early effectors

mediating destructive events involved in synaptic dismantlement. ROCK 2 was recently isolated in a proteomic and genomic screen aimed at isolating molecular effectors involved in synaptic degeneration [45]. It is thus tempting to propose that inhibitors of ROCK could represent interesting pharmacological compounds for further translational development.

Conclusions

Microfluidic devices have facilitated investigation in biology in new ways, thanks to the structuring of the *in vitro* model, micro-environment control, and design flexibility. This work, which follows the trend of “organ on chip” research, shows that microfluidic platforms for nerve pathway reconstruction are useful tools for the study of both developmental and degenerative processes at several levels, ranging from sub-cellular compartments to integrated neuronal networks. Here we show that the use of this new sophisticated but still simple to handle *in vitro* reconstructed neuronal network not only allows basic biological questions to be addressed but also represents a valuable and relevant tool for fast screening of synapto-protective drugs. Using this strategy, it has been possible to reveal for the first time that Rho kinase may represent interesting targets to delay synaptic collapse after injury. If this could speed up and improve the reliability of the validation and characterization stages when developing therapeutic drugs, this would already be a great step forward.

Supporting Information

Figure S1 Automated quantification of synapses with SynD software. Synaptic disconnection was assessed by counting v-GLUT1 clusters affixed to MAP-2 positive striatal

dendrites. Images were all obtained with the same acquisition parameters and were similarly processed with ImageJ software before being used for quantification. (a) v-GLUT1/MAP-2 merge used for quantification using SynD software. (b) Automatic soma detected using thresholding followed by morphological opening and closing. (c) Dendrites detected using steerable filters. A cost function evaluates each pixel around the current neurite mask, and those with a cost lower than a specified threshold are added to the mask. The cost function takes into account how neurite-like the neighborhood around the candidate pixel is (i.e. ideally a bright line segment surrounded by dark regions on either side), and if the candidate pixel is in the direction of the already detected parts of the neurite. (d) The detected neurite mask overlaid on the v-GLUT1 channel, only the parts located either within the neurite mask or a small neighborhood around it are used for synapse detection. (e) Synapse regions are detected by thresholding the v-GLUT1 channel; putative centers are located by deconvoluting the image. (EPS)

Acknowledgments

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Author Contributions

Conceived and designed the experiments: JMP BB BS JLV. Performed the experiments: BD BL MV VS AAB. Analyzed the data: BD BL VS AAB JJH. Contributed reagents/materials/analysis tools: AAB BS. Wrote the paper: BD JMP BS BB JLV.

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Chapter B

FDV bachelor students: promotion 2011 and 2012

Promotion 2011

In alphabetical order:

Pierre Arcin
Fanny Bernardon
Perla Bokobza
Maxime Borry
Théotime Calandra
Aimen El Assimi
Lydiane Gantier
Ioana Genescu
Morgane Grand
Isabelle Hédon
Lisa Jacquy
Lucy Kundura
Léa Laffond
Christine Lazarescu
Maité Marques
Ramine Mecheri
Emeline Oueda Cruz

Maria Pothier
Asma Safsaf
Guillaume Villain

Promotion 2012

In alphabetical order:

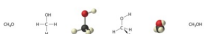
Julien Barrere
Kilian Basto
Clovis Basier
Salomé Bourgeau
Ivan Cornut
Léna Coutrot
Louis Faure
Quentin Ferdinand
Anton François
Magali Hanot
Nicolas Lafosse
Julie Le Naour
Juliette Lenouvel
Benjamin Le Sant
Théo Pierre
Rebecca Richard
Pierre-Luc Satin
Derya Sebukhan
Axel Viala
Amaury Vincent

Chapter C

Surveys used in the pedagogy study

Pre-game and Post-game survey on the Lewis game

1.

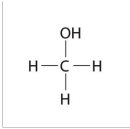


Do you think these pictures are different representations of the same molecule or representations of several molecules?

A. Different representations of the same molecule.

B. Representations of several molecules.

2.



Are you familiar with this representation of methanol?

A. Yes.

B. No.

3.

Pick randomly one of the following answers.

A. 1

B. 2

C. 3

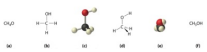
4.

Do you know about Lewis representation of molecules?

A. Yes.

B. No.

5.



Classify in the same order the tag of the molecule(s) representations (a<b<c<...<f) and the associated names of the different representations of the molecule(s) of the image.

• Ball and stick model

• Molecular Formula

• VSEPR Structure

• Space filling model

• Lewis Structure

• Condensed structural formula

6. As the temperature increases, what happens to the kinetic energy (KE) of the gas molecules?

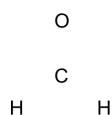
A. KE increases

B. KE decreases

C. KE stays the same

7.

Draw as many lines as needed to form the covalent bonds of the methanal molecule above.



8.

Ice and steam are both water. In which state are the water molecules the largest?



- A. The ice, because water expands when it freezes.
- B. The steam, because gas molecules are small.
- C. All water molecules are the same size.
- D. Not enough information.

9.

For the best Lewis diagram, how many lone pairs of electrons are there around sulfur in SF₄? (best is defined as having the minimum formal charge on all atoms)

- A. 0 lone pairs
- B. 1 lone pair
- C. 2 lone pair
- D. 3 lone pair

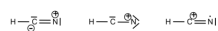
10.

For the atom K, the mass number is 40 and the atomic number is 19. How many neutrons are there?

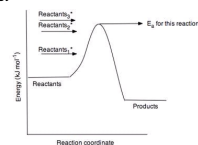
- A. 40
- B. 19
- C. 21

11.

Click on the formal charge which is not properly attributed.



12. The activation energy is the critical collision energy required for a pair of molecules to react Which reactants have enough activation energy?



- A. Activated Reactants 1
- B. Activated Reactants 2
- C. Activated Reactants 3

13.

Write down your definition(s) of what is an atom.

14.

According to VSEPR theory, what is the geometry of the methane molecule ?

- A. linear
- B. tetrahedric
- C. hexagonal
- D. spherical

15.

For the molecule Hydrogen Cyanide, what is the arrangement of the atoms in the molecule?

- A. H-N-C
- B. N-H-C
- C. H-C-N
- D. Have no clue

16.

Rank the electronic configuration below in the same order as the atoms classified below: O (Z=8) < Li (Z=3) < Cu (Z=29) < Mg (Z=12)

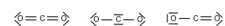
- [Ar] 4s(1) 3d(10)
- 1s(2) 2s(2) 2p(4)
- 1s(2) 2s(2) 2p(6) 3s(2)
- 1s(2) 2s(1)

17.

Write down your definition(s) of what is a molecule.

18.

Click on the right and most stable Lewis representation of the CO₂ molecule (click on one of the atoms of the right molecule to make sure it will be counted as the right answer).

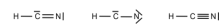


500 mL of water were added to a 1 L container, which was sealed and held at 25°C. What phase(s) are present at equilibrium?

- 21.

Figure 1 shows a 10x10 grid representing a 10000-dimensional input space. The grid is divided into four quadrants by a vertical line at column 5 and a horizontal line at row 5. The top-left quadrant (rows 1-5, columns 1-5) is colored blue. The top-right quadrant (rows 1-5, columns 6-10) is colored green. The bottom-left quadrant (rows 6-10, columns 1-5) is colored yellow. The bottom-right quadrant (rows 6-10, columns 6-10) is colored red. The grid is labeled with 'x' and 'y' axes, with 'x' ranging from 0 to 9 and 'y' ranging from 0 to 9.

Click on the right and most stable Lewis representation of the HCN molecule (click on one of the atoms of the right molecule to make sure it will be counted as the right answer).



Pre-game survey on the VSEPR game

1.

Have you already heard about VSEPR representations or theory?

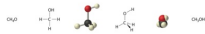
- A. Yes and I master it
- B. Yes I am familiar with it
- C. Yes a little bit
- D. Not at all

2. Neurotransmitter release and binding causes what to happen to the postsynaptic Vm?

- A. Increase
- B. Decrease
- C. Could be either
- D. Neither Increase nor decrease

3.

Click on the VSEPR representation of the molecule CH₄O.



4.

Click on the block p of this periodic table.

Periodic Table of the Elements



5.

What VSEPR stands for?

- A. Valence Shell Electrons Pair Repulsion
- B. Valence Spin Electronic Pair Repulsion
- C. Valence Shell Electrons Pair Representation
- D. Valence Shell Electronegativity Parity and Repulsion

6.

What VSEPR is useful for?

- A. Describing the electronic pairs repartition around an atom
- B. Representing molecules in 3D
- C. Describing the geometry of molecules
- D. Understanding steric effects in chemical reactions

7.

Which sentence best describe the photoelectric effect?

- A. Interaction between light and materials.
- B. Extraction of electrons from atoms by the effect of light.
- C. Effect in which photons tranfer their energy to electrons.
- D. Effect understood by Einstein.

9.

Click on one element of this table which presents 5 valence electrons.

Periodic Table of the Elements

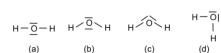
11.

The type of molecular interactions that Glycine (R=H) and alanine (R=CH3) are capable of are:

- A. Hydrogen bonds
- B. Ionic interactions
- C. Van der Waals
- D. Acid/base interactions

8.

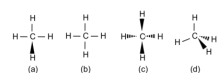
What is the correct 3D representation of the H2O molecule?



- A. a
- B. b
- C. c
- D. d

10.

What is the correct 3D representation of the CH4 molecule?



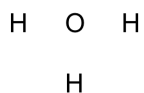
- A. a
- B. b
- C. c
- D. d

12.

Which is the fundamental and basic principle underlying VSEPR theory?

13.

Draw the Lewis structure of the H_3O^+ ion. Don't forget lone pairs and charges.

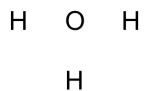


14. What is required in the axon terminal to start neurotransmitter release?

- A. Increased intracellular Na generated during an AP
- B. Decreased intracellular K generated during an AP
- C. An increase in membrane potential generated during an AP
- D. All of the above

15.

Draw the VSEPR (or 3D) structure of the H_3O^+ ion.



16.

As the wavelength of the wave increases, the frequency of the wave:

- A. Increases
- B. Stays the same
- C. Decreases
- D. None of the above

17.

Describe the steps (around 4 steps) which allow you to build the 3D representations of molecules by following VSEPR theory.

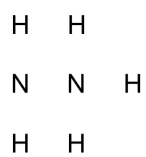
18.

If the ligands are on the x, y and z-axis, which d-orbitals will be more affected?

- A. d_{xy} , d_{yz} and d_{xz}
- B. $d_{x^2-y^2}$ and d_{z^2}
- C. both are equally affected

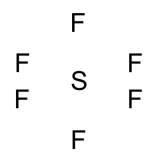
19.

Draw the Lewis structure of the N_2H_5^+ ion. Don't forget lone pairs and charges.



20.

Draw the 3D representation of SF_6 .



Post-game survey on the VSEPR game

1.

Have you already heard about VSEPR representations or theory?

- A. Yes and I master it
- B. Yes I am familiar with it
- C. Yes a little bit
- D. Not at all

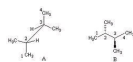
2.

A photon of which type of light has the lowest energy?

- A. infrared light
- B. visible light
- C. x-rays

3.

Click on the VSEPR representation of the molecule.



4.

Click on the block s of this periodic table.

Periodic Table of the Elements

Click on the block s of this periodic table.



5.

What VSEPR stands for?

- A. Valence Shell Electrons Pair Repulsion
- B. Valence Spin Electronic Pair Repulsion
- C. Valence Shell Electrons Pair Representation
- D. Valence Shell Electronegativity Parity and Repulsion

6. Similar to neurons, astrocytes can be depolarized. What ions are responsible for their depolarization?

- A. Sodium
- B. Potassium
- C. Calcium
- D. Chloride

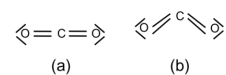
7.

What VSEPR is useful for?

- A. Describing the electronic pairs repartition around an atom
- B. Representing molecules in 3D
- C. Describing the geometry of molecules
- D. Understanding steric effects in chemical reactions

8.

What is the correct 3D representation of the CO₂ molecule?



- A. a
- B. b

9.

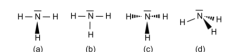
Click on one element of this table which presents 5 valence electrons.

Periodic Table of the Elements



10.

What is the correct 3D representation of the NH₃ molecule?



- A. a
- B. b
- C. c
- D. d

11.

The type of molecular interactions that Cystein (R=CH₂-SH) and Serine (R=CH₂-OH) are capable of are:

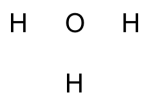
- A. Hydrogen bonds
- B. Ionic interactions
- C. Van der Waals
- D. Acid/base interactions

12.

What is the most fundamental rule of VSEPR theory?

13.

Draw the Lewis structure of the H_3O^+ ion. Don't forget lone pairs and charges.

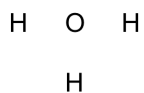


14. What is required in the axon terminal to start neurotransmitter release?

- A. Increased intracellular Na generated during an AP
- B. Decreased intracellular K generated during an AP
- C. An increase in membrane potential generated during an AP
- D. All of the above

15.

Draw the VSEPR (or 3D) structure of the H_3O^+ ion.



16. How many wavelengths are shown here?



17.

Describe the steps (around 4 steps) which allow you to build the 3D representations of molecules by following VSEPR theory.

18.

Which d orbitals will be affected the most in a tetrahedral geometry?

- A. The d orbitals off axis
- B. The d orbitals on-axis

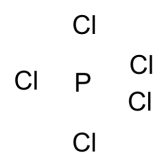
19.

Draw the Lewis structure of the H_2O_2 . Don't forget lone pairs and charges (if needed).



20.

Draw the 3D representation of PCl_5 .



Final survey on the Lewis and VSEPR game

1.

How would you define your knowledge about Lewis representations and theory?

- A. I master it
- B. I am familiar with it
- C. I know a little bit about it
- D. I don't master it very well

Question 21833

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2.

How would you define your knowledge about Cram representations and VSEPR theory?

- A. I master it
- B. I am familiar with it
- C. I know a little bit about it
- D. I don't master it very well

Question 22760

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3.

In the following ways of learning, which one do you think is your favorite at the moment ?

- A. Interactive frontal course
- B. Self-learning
- C. Learning through games
- D. Learning through projects
- E. Learning through practical courses
- F. Learning in internships

Question 22765

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4.

How much did you like the games used for teaching in chemistry ? Give me your answer on a scale from 1 (you disliked it) to 5 (you appreciated it a lot).

- A. 1
- B. 2
- C. 3
- D. 4
- E. 5

Question 22761

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5.

How much do you think these games helped you in learning the course ? 1 (not at all) and 5 (a lot).

- A. 1
- B. 2
- C. 3
- D. 4
- E. 5

Question 22762

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6.

How much do you think these games helped you in understanding the course ? 1 (not at all) and 5 (a lot).

- A. 1
- B. 2
- C. 3
- D. 4
- E. 5

Question 22763

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7.

Among the three games we played, which one did you like the most ?

- A. Game on Lewis
- B. Game on VSEPR
- C. Qui est-ce des éléments

Question 22764

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8.

Draw the Lewis structure of the H₂O₂. Don't forget lone pairs and charges (if needed).

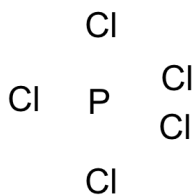


Question 21875

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9.

Draw the 3D representation of PCl₅.

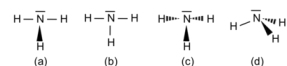


Question 21874

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10.

What is the correct 3D representation of the NH₃ molecule?



- A. a
- B. b
- C. c
- D. d

Question 21872

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11.

Which sentence best describe the photoelectric effect?

- A. Interaction between light and materials.
- B. Extraction of electrons from atoms by the effect of light.
- C. Effect in which photons transfer their energy to electrons.
- D. Effect understood by Einstein.

Question 21854

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12.

What is the most fundamental rule of VSEPR theory?

Question 21851

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13.

Click on one element of this table which presents 5 valence electrons.

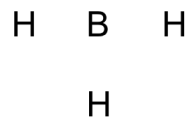
Periodic Table of the Elements

Question 21840

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14.

Draw the Lewis representation of BH₃.



Question 21693

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15.

Considérons donc l'ion polyatomique (ou molécule chargée) HO⁻. Compter le nombre d'électrons de valence et de doublets de valence.

- A. 7 électrons de valence et 4 doublets.
- B. 7 électrons de valence, 3 doublets et un électron célibataire.
- C. 8 électrons de valence, 4 doublets.
- D. 8 électrons de valence, 3 doublets et 2 électrons célibataires.

Compter d'abord les électrons de valence de chaque atome (neutre) pris séparément et ensuite ajouter la charge globale à votre décompte.

Question 21689

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16.

What is an atomic orbital ?

Question 16431

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17.

Draw the Lewis structure of N₂.

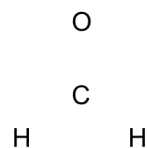


Question 21668

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18.

Draw as many lines as needed to form the covalent bonds of the methanal molecule above.



Question 21470

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19.

Write down your definition(s) of what is an atom.

Question 21462

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20.

What VSEPR is useful for?

- A. Describing the electronic pairs repartition around an atom
- B. Representing molecules in 3D
- C. Describing the geometry of molecules
- D. Understanding steric effects in chemical reactions

Question 21837

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21.

Write down your definition(s) of what is a molecule.

Question 21460

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22.

How would you define a covalent bond ?

- A. an unbreakable link between two atoms
- B. a sharing of two electrons between two atoms
- C. a breakable link between two atoms
- D. one of the component of any molecule

Question 16432

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Survey on the Nucleo-Belote game

[Modifier ce formulaire](#)

Electrophile/Nucleophile game survey

***Obligatoire**

If you consider the carbon of the carboxylic acid group, would you say this atom is electrophile or nucleophile? *

- ☐ electrophile
- ☐ nucleophile
- ☐ I don't know

According to you, what is the most nucleophile between an alcohol group and bromide ion? *

- ☐ alcohol
- ☐ bromide ion
- ☐ I don't know

Did you like playing the game? *

- ☐ Yes
- ☐ No

Did you know what was an electrophile and a nucleophile before playing the game? *

- ☐ Yes
- ☐ No

Was the game fun? *

- ☐ Yes a lot
- ☐ Yes a little
- ☐ Not really
- ☐ Not at all

Do you have the feeling you learned something through the game? *

- ☐ Yes a lot
- ☐ Yes a little
- ☐ Not really
- ☐ Not at all

If yes, what did you learn...

Do you have the feeling the game helped you to learn what is an electrophile and/or a nucleophile? *

- ☐ Yes a lot
- ☐ Yes a little
- ☐ Not really
- ☐ Not at all

If yes, how did it help you...

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Résumé

Ma thèse comporte deux grandes parties, la première en biophysique et la seconde en pédagogie. La première partie présentent des travaux à la frontière entre neurobiologie et microfluidique. Le but de ces travaux est de pouvoir reconstruire et étudier des réseaux complexes de neurones *in vitro* avec une topologie de connexions synaptiques bien contrôlée. Une série de micro-structures mécanique et/ou chimique ont été étudiées pour leur capacités à (i) positionner les corps cellulaires des neurones, (ii) orienter la pousse des neurites, et (iii) différencier les axones des dendrites. Un premier réseau comportant trois populations de neurones connectées en série a été reconstruit à l'intérieur d'un circuit microfluidique. Ce réseau qui mime la voie perforante de l'hippocampe pourra être exploité pour des études en physiologie ou en neuro-dénérescence. Une méthode entièrement optique de stimulation et d'observation de l'activité neuronal a été mise au point. Elle ouvre de nouvelles portes pour étudier des processus cognitifs complexes dans des systèmes simplifiés *in vitro*. La seconde partie présente le développement et l'étude de jeux pédagogiques pour enseigner les bases de la chimie organique. Ces jeux, qui peuvent selon les cas remplacer un cours ou une séance d'exercices, semblent intéressant pour aider à la compréhension et à la mémorisation de concepts tels que la géométrie des molécules ou la réactivité entre molécules organiques.

Summary

My PhD is divided in two parts, one in biophysics and one in pedagogy. The first part present results at the frontier between neurobiology and microfluidic. The overarching goal of this work was to develop tools and methods to build and study complex neuronal networks controlling the topology of synaptic connexions. Micro-patterning techniques with mechanical and/or chemical constraints were explored regarding their capacity to (i) position cell bodies, (ii) orient neurite outgrowth and (iii) polarize neurons. For the first time, a network comprising three different neuronal populations connected in specified directions was reconstructed in a microfluidic device. This network that mimics the perforant pathway of the hippocampus can be used to study physiological rythms or neurodegenerative processes including Alzheimer's disease. A novel and fully optical method is presented to stimulate and record neuronal activity *in vitro*. It opens new routes to study complex cognitive processes in simplified *in vitro* systems. The second part of my work present the development and assessment of educational games to teach chemistry at the undergraduate level. These games can either be used to replace courses or exercises. They seem promising to improve the understanding and memorization of chemistry concepts such as molecular geometry and organic reactivity.