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Thèse de doctorat – spécialité Physique

Emergent design of neuronal devices

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Emergent design of neuronal devices

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À mes parents.

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General introduction

Being able to harness the processing power of biological neurons will open the door to ground breaking applications in many scientific fields. Glucosepowered neural implants could repair or enhance cerebral functions in the field of medicine, *in vitro* cognitive assays could foster drug screening in the pharmaceutical industry, and biological processors could lead to new kinds of artificial intelligence.

Any neuronal culture assigned with a computable function (actual or intended) can go under the name of a neuronal device. This thesis represents a multi-frontal effort towards making this concept a more tangible thing, without holding to firmly onto preconceptions of what might and might not be relevant for computation. Our strategy is based on the exploitation of cultured neuronal networks' properties that are both resilient and experimentally easily accessible rather than on the urge to reproduce the machinery of the brain.

This seemingly naive engineering approach is disputable in the same way chance and necessity are in general disputable in biology. In spite of their mindboggling capabilities, one can argue that brains are the product of historical contingency and are therefore not necessarily optimal for the functions they support *in vivo*. This would be even more so the case for alternative functions only relevant to disembodied cultures.

Whereas it might then be defendable to distance ourselves from natural brains, there are several benefits to the use of living components in the first place. Living cells have evolved to be energetically very efficient, they can repair themselves and adapt to environmental changes. On a more fundamental level, *in vitro* discoveries, originating from the making and characterization of neuronal devices, might be transposable to processes at play *in vivo* but difficult to access; such undertaking is therefore also relevant to the field of neurosciences in general. Finally and most importantly, this work could help to define the kind of computations that can be performed by neuronal assemblies and bring new elements to the field of artificial intelligence, which is bound to be determinant in our future.

Intelligence, the brain and the neurons

While many different definitions of intelligence are found in the literature [1], it can in a very general way be seen as the ability to take "educated" decisions in various situations to maximize an objective function. An intelligent agent should therefore be able to :

- collect information from its environment.
- process this information into decisions (follow a policy).
- express its decisions.

Intelligence is a relative concept which can only be quantified given a particular objective function. The objective function that an intelligent system is ought to maximize can be virtually anything, and is usually organized into intermediate functions. For instance in animals, the global objective function is gene transmission, which is a corollary to natural selection, and it is maximized through more elementary objectives including survival, mating, offspring protection, down to the level of individual moves.

If the great lines of this objective function are necessarily similar in all living organisms and quite simple to understand, the way it is internally organized can be very different across species and give rise to a panel of elaborate and complex exotic behaviors.

However intuitively we don't interpret all complex behaviors as signs of intelligence. Another concept that we often associate with high intelligence and that we have not mentioned yet is at play : learning. In this framework, learning is simply the process through which a system becomes *more* intelligent. A learning agent would additionally require the ability to :

- judge its own actions.
- change its "decision-making policy" to increase the value of future decisions.

Giving a system the ability to learn so that it can develop policies relevant to new situations, or even new objectives, is more parsimonious than trying to cover in advance all eventualities, which becomes quickly impracticable as the set of possible situations and actions grow larger. Learning is the only way to be good at a non-limited set of tasks, and is therefore critical to the development of high-level and general intelligence (the kind humans have).

Although it is known for a long time that the brain is the organ that allows intelligence and learning, how these properties emerge from it remains elusive. By taking a rational materialistic position however, intelligence *must* be the sole result of the processes occurring in the brain ; regardless of its complexity, the brain remains a physical machine obeying the natural laws of the universe. And since we have at least one example of a living machine that can create human-level intelligence (the brain), there is no reason to doubt that other machines, yet to be invented, can perform equally or actually even better.

Indeed, because evolution works only through non destructive gradual changes (or reappropriation) of preexisting structures it cannot guarantee global optimality, but merely solutions that work "well enough". The absence of *tabula rasa* in evolution can actually lead to ludicrous designs, like the backward retina of vertebrates which yields an otherwise easily avoidable blind spot, or the laryngeal nerve of giraffes that makes a useless detour of several meters to connect regions only few centimeters apart (Fig. 1).



FIGURE 1. – **Sub-optimality in natural systems.** A- The laryngeal nerve originating from the brain takes in the case of giraffes an almost 5 meters long detour around the heart before it connects to the larynx, only few centimeters away from the starting point (Credit : Adapted from illustration by Vladimir V. Medeyko). B- In vertebrate eyes (left), the nerve projections route before the photosensitive retinal cells, blocking some light and creating a blind spot where the fibers pass through the retina out of the eye ball. A better design would be to simply flip the retina, like it is the case in the octopus eyes (right), where the nerve fibers route behind the retina, and do not block light or disrupt the retina. 1-Retinal cells. 2-Nerve fibers. 3-Optic nerve. 4-Blind spot. (Credit : adapted from an illustration by Jerry Crimson Mann)

If there is no good reason to stick to a particular brain architecture, and given the opportunity to build an artificial brain from scratch, what would be the best way to do it? Despite some inelegant designs, it is noticeable that all brains across the animal kingdom share the same basic functional constituent, the neuron. It would probably be a bad idea to ignore this striking success. Plus, we have to recognize that as far as intelligence is concerned, there is not many other sources of inspiration in nature.

Neurons are specialized cells found in the nervous system of every animal on the planet and assume, despite some variations, a typical morphology (Fig. 2). Neurons have a small (typically 15-50 microns) cell body from which extend thin processes, or neurites, through which they can contact their neighbors. Typically, neurons have two types of neurites : dendrites, that branch in a dense and intricate tree, and a single axon, that can grow over very long distances before splitting into a terminal arborization to contact other cells. Those specialized contacts are called synapses, and mediate signals in a directed manner, from the axon of one neuron, to the dendrites of an other neuron. The structural features of neurons are understandable in light of the functions they support *in vivo*. The dendritic tree offers a large surface for synapse formation and signal integration. The cell body provides an operating point on which received signals are processed into an output. Finally the axon constitutes a specialized route for the delivery of the output to distant targets.

The most important functional feature of neurons is that they are electrically excitable, meaning that some processes inside them are coupled together via a electric potential existing across the cell membrane.

Like all cells, neurons possess a membrane which separates the intracellular medium from the outside. Because cells actively maintain a differential repartition of charged species between the intracellular and extracellular compartments, there is an electric potential difference, or voltage, across the membrane. At rest, the intracellular compartment sits at about -65 mV from the extracellular potential. However the membrane potential is constantly modulated by transient ionic currents across the membrane.

Ionic currents are controlled by the opening of ion channels present in the membrane. Ions spontaneously diffuse through open channels, following a combination of electrostatic forces and concentration gradients called the electrochemical potential. Different effectors can open these channels, including neurotransmitters released by other neurons, external factors (such as light, odorant molecules, mechanical forces), or even the membrane potential itself.

In the latter case, voltage sensing is achieved by membrane proteins that can adopt different conformations, hence functional properties, depending on the electric field they experience. Those sensors are usually ion channels themselves and can switch between high and low conductance states.

The opening of voltage-gated ion channels can trigger different processes inside the cell. Most notably, secretion of neurotransmitters at synapses is modulated by calcium ions flowing inside the cells through voltage-gated calcium channels. More importantly, the fact that opening of voltage-gated channels affects in turn the membrane potential allows positive and negative feedback loops, which are key to a fundamental mechanism taking place in almost all neurons : the action potential, or spike.

Spikes are electric impulsions (Fig. 3) supported by two main types of voltage-gated ion channels which open upon membrane depolarization. When the membrane potential increases to about -50 mV, closed sodium-gated ion channels, mostly located at the base of the axon, open and sodium rushes



FIGURE 2. – **Neuronal morphology and variability.** Top : Principal components of a neuron, showing the flow of information from dendrites to the axon terminal. Bottom : Different types of mammalian neurons showing high morphological variability. 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 Ramón y Cajal)

into the cell following its electrochemical potential. Since sodium ions are charged positively, the inwards flow depolarizes the membrane even more, which triggers the opening of adjacent channels. The depolarization quickly spreads along the axons (up to 120 m/s in some nerve fibers) and stops when sodium channels enter transiently into a third state called the inactivated state. The inactivation of sodium channels is responsible for an absolute refractory period, lasting about one millisecond, during which it is impossible to initiate another action potential.

In parallel, this depolarization induces the opening of voltage-gated potassium channels, albeit with a small delay. Following their electrochemical potentials, potassium ions quickly diffuse out of the cell. This outward flow of positive charges repolarizes the membrane. However, because the potassium channels do not close immediately after the membrane has returned to its resting potential, a transient hyper-polarization, or overshoot, can be observed. This hyper-polarization makes it harder to reach the threshold immediately after an action potential has taken place, which gives rise to a relative refractory period, and helps guaranteeing an unidirectional propagation of action potentials only towards the not yet excited portions of the axons.

Back to the resting potential, the inactivated sodium channels become excitable again and another action potential is therefore permitted. The cycle is completed by a specialized pump called sodium/potassium-ATPase which actively translocates sodium and potassium ions that flow in and out of the cell during action potentials back to their original compartments. Although at the molecular level the opening and closing of channels is stochastic, the collective averaged behavior of the channels insure the generation of very reproducible spikes.



FIGURE 3. – Action potentials and voltage gated ion channels. Top : evolution of membrane potential during spikes showing the depolarization phase, the repolarization phase and the overshoot . Bottom : average state of sodium and potassium channels during spikes. The colors transcribes the distribution of the channel across the different states (e.g. yellow means that 50% of the channels are closed (red) and 50% are open (green))Sodium channels open abruptly when the threshold voltage is reached and inactivate immediately after which creates an acute depolarization. The delayed opening of potassium channels repolarizes the membrane while the sodium channel are still inactivated. The delayed closing of those same channels create the overshoot.

The ability to generate spikes gives rise to different properties that shaped the evolution of life. It allows fast propagation of signals over long distances and without dissipation, hence movement coordination and locomotion in larger organisms. The all-or-none nature of action potential is also critical in the way information is encoded and processed in the brain.

Coding and processing of information in the brain

Although we do not want to strictly limit ourselves to what happens to be used in nature, we will review in this part how information is coded and processed *in vivo* by the intended building blocks of our neuronal devices.

The binary nature of action potentials means no information is possibly encoded in their amplitudes, and the consensus is therefore that all brain activity is recapitulated only by the time points at which the spikes of each neurons happen. However there is no consensus regarding the way we should read these spikes, that is, the way information is encoded in them.

One of the first proposed hypothesis was that neurons could encode information in their average firing rate (number of spike per second, Fig. 4 top), which would allow transmission of pseudo-analog signals in the nervous system. This kind of code was in fact rapidly demonstrated to be relevant *in vivo*, to represent for instance the level of activation of stretch receptors inside muscles [2]. This paradigm is still strong today, in particular because it happened to work fairly well in artificial neural networks algorithms.

However there are good reasons to believe information is not universally encoded in this manner. For instance, observation of spike trains in the brain usually show very irregular spike patterns, which does not make sense if evolution optimized neurons for pure frequency coding. Secondly, accurately averaging low firing rates would require, for single neurons, durations that living animals can not afford in a context where they need to respond quickly to their environments. Finally there are evidences that complex visual scene analysis can be achieved in a single feed-forward pass of under 150 ms in the human brain [3]. This would give time for neurons in the different layers of the visual system to fire only once, ruling out the rate code hypothesis.

Theoretical and experimental evidence have in parallel accumulated in favor of temporal coding. Since there is no absolute time reference in the brain, we can expect temporal coding to assume different shapes.

In the case of a punctual time reference, the information would be encoded in the delay between the spike and the reference. It has been shown that the onset of a stimulus can play the role of such reference [4, 5], although because external stimulation always translate into neuronal activity, timing between spikes is proximally what matters (Fig. 4 middle). It was demonstrated in some cases that the latency of the first spike to occur after the reference carries most of the information, but that the presence of subsequent spikes, independently of their precise timing, also matters [6]. Ongoing coarse-grained cyclic activity, like the different brain waves, can also be a relevant time label. In a case where the reference is cyclic, the information would be encoded in the phase of firing (Fig. 4 bottom). In particular, the place cells found in the hippocampus which fire when a rat enters a specific area, do so with a phase, relative to the intrinsic theta rhythm, which depends on its precise location within the area. In some study [7], researchers could significantly refine the predicted location of the rat by taking into consideration the phase of firing of place cells, in addition to their firing rate.

From these studies we know at least one thing for sure : there is no universal neural code. Evolution produced different strategies to code different types of information, sometimes multiplexing different codes onto a same neuron without any consideration, unfortunately, for making it intelligible to us.



FIGURE 4. – **Different possible coding schemes.** Spikes are represented as small vertical bars. The red bars are reference spikes. The red curves represent intrinsic oscillations.

As far as computation is concerned, we can consider, as a first approximation, that the processing capabilities of a single neuron are rather dull. It integrates signals from incoming connections and if the sum reaches a threshold value it sends in turn a signal to its outputs. Intuitively, a threshold activation function makes sense for decision making. Such simple thresholding seems sufficient, when implemented in artificial neural networks, to perform complex classification tasks provided that the connections between neurons, that is, the strength of the synapses, are properly tuned.

However, the processing capabilities of individual neurons have been suspected for a long time to be significantly richer in light of the very typical dendritic architectures of some neuronal types. The discovery of voltage-gated ion channels and action-potentials inside the dendrites [8, 9] obliterated the idea that they merely funnel incoming signals passively to the soma. Interestingly it was shown that under particular conditions, dendritic spikes can compensate for the distance-dependent attenuation observed in passive dendrites, so that the efficacy of synapses is nearly location-independent [10]. The function of active dendrites could therefore simply be a way for neurons to free themselves from the strong metric constraints inherent to physical networks. But a neuron endowed with excitable dendrites could do much more, and perform a complex non-linear summation of its synaptic inputs, depending on their spatial and temporal activation pattern. If the importance of active dendritic trees has been predicted for a long time, their roles in computation has been experimentally observed quite recently. In the retina for instance, direction selectivity was shown to depend on active dendritic processing [11].

Interestingly, theoretical investigations [12] showed that under certain circumstances even neurons with passive, non excitable dendrites can compute non trivial functions (like the XOR logic gate), so that dendritic (pre)processing might be a general feature throughout the nervous system rather than an exception. In practice it might not be evident to exploit such computing power in artificial devices because it would presumably require precise positioning of synapses on the dendritic tree of each neuron, which is firstly technologically challenging and secondly intrinsically regulated through non yet elucidated mechanisms.

Fortunately, complex dendritic processing happening inside special neurons can be emulated by networks of simpler neurons, so that complex computation is always achievable as long as we have enough neurons and that we can tune the weight of the different connections in the network appropriately.

The power of neuronal computation takes indeed its real dimension when neurons come together. Having a lot of neurons is useful by itself in order to solve several coding problems, like rapid low-rate encoding (over several neurons instead of several seconds) and noise reduction[13]. But having a lot of neurons is not enough. In the human brain we can see that not only 86 billions neurons are packed together but also that they are connected together in a unfathomably complex, non random way.

The circuitry of the different functional blocks of the brain have become specialized, along the neuronal code, to perform very different tasks, yielding different connectivity patterns across the nervous system. Sometimes, like in the vertebrate's retina, the physical connectivity can be easily linked to the type of processing going on.

For example, edge detection in the visual field can be supported by an array of simple modules comprising a patch of photoreceptors, one horizontal

cell and one bipolar cell. Whereas the bipolar cell receives a single excitatory input from the photoreceptor located in the center of the patch, the horizontal cell receives many weak excitatory inputs from all the photoreceptors, and send inhibitory projections back at them (Fig. 5).



FIGURE 5. – **Structure and function of the retina circuitry.** P=Photoreceptor. H=Horizontal cell. B=Bipolar Cell. Gray level in the cells represent the degree of activation, white being the most activated (depolarized). Yellow background represents illumination, and purple background represent dark areas. A- Inhibitory feedbacks from the activated H cell prevent all photoreceptors to be strongly depolarized at the same time. B- The photoreceptors are inactive under dark conditions. C- When only one photoreceptor is in the dark, H remains silent and the photoreceptor can reach the level of excitation necessary to activate B.

Under global dark conditions, the photoreceptors are activated (depolarized) by intrinsic sodium currents but this activation is limited by inhibitory feedbacks from the horizontal cell (which is strongly activated by all the photoreceptors) and the bipolar cell is therefore not responding (Fig. 5 A). Under global illumination, the photoreceptors are inactivated (hyper-polarized) too as the phototransduction cascade reduces the sodium currents; the bipolar cell is not responding either (Fig. 5 B). When only one photoreceptor is in the dark, the inhibitory feedback from the horizontal cell in negligible (because it receives only one weak excitatory input), and the photoreceptor becomes strongly depolarized. If this photoreceptor is the one in the middle of the patch, then it will send a strong excitatory signal to the bipolar cell which will become activated (Fig. 5 C). The bipolar cell depicted here is labeled OFF-center because it is activated by a dark center and a bright surround. Other circuits allow the emergence of ON-center bipolar cells, which together with OFF-center bipolar cells transmit to downstream structures a picture which has enhanced contours (Fig. 6). Additional filtering and labeling continues, carried out by more and more complex feature detectors, up to the visual cortex and beyond.



FIGURE 6. – **ON and OFF-center processing enhances contours.** A-Original gray scale picture of a a molting cicada. B- Activity of on and off-center filters are shown in green and red respectively.

In other cases, understanding the function of neural circuits might not be as "simple". It is known for half a century that the neocortex of the mammalian brain also presents a very typical, stratified, architecture [14]. Closer examination reveals that it is organized in modules of a few tens of neurons, distributed over the different cortical layers, called neocortical mini-columns [15]. These modules are thought to perform very general purpose computations, evidenced for instance by the fact that the visual cortex can be recycled, in blind people, to treat additional auditory and somatosensory information [16]. In a great effort, the Blue Brain Project has been integrating in a giant simulation numerous anatomical and physiological data of the different neuronal subtypes found in the neocortex [17]. Despite such simulation could already reproduce some activity patterns recorded *in vivo*, there is no way to determine if the simulated network actually computes anything useful yet. Currently there is still no functional model of the cortical mini-column yet, and investigations continue [18, 19].

Finally, other circuits in the brain have been hypothesized to simply decode and re-encode information between the different computational blocks [20] of the brain. Those few examples show that for a given number of neurons, there are as many possible functions that there are ways of connecting them together. The question of how nature manages to robustly and accurately build such large networks brings us to the next point.

The information required to completely determine the connectivity matrix of a network the size of our brains, even if we only considered the mere presence of connections and not also their strengths, would be a humongous 10 billion terabits (n^2 bits, with n=100 billions neurons). Supposing a perfect -surrealistic- information coding scheme in the DNA, the human genome would at best encode less than 1% of a terabit (2 bits for each of the 3 billion base pairs). Even if these approximations are bold, there is enough margin to say with confidence that the genes can only account very partially for the connectome of the brain and that additional information has to come from elsewhere.

The fact that the brain is embedded in a metric space removes many degrees of freedom in the connectivity matrix. The distance between neurons is for instance a strong determinant for connectivity. It has been hypothesized [21] that canonical connectivity in the brain is, at least locally, the result of the initial positioning of the cell somas on one hand, and the shape and extent of their arborizations on the other end. In this scenario, the resulting dendrite/axon overlap is what eventually determines the connectivity. Of course, the positioning of the cells, as well as their shapes, are not purely genetically determined either, and result of interactions between intrinsic growth processes and physical constraints (the brain has to grow inside a limited volume for instance). Physical and genetical determinants of morphogenesis were investigated in other biological systems here in our laboratory (MSC). In particular, it was shown that the shapes and folds of leaves are as much the result of constrained growth inside a finite volume (delimited by the bud) as they are the product of gene expression [22].

At this point, the resulting canonical connectivity would not account for behavioral differences observed between individuals of the same species living in different environments. Since animals have to live in a world which is susceptible of change from one generation to the other, it makes sense to use the extra-information drawn from it (visual, auditory, sensory) to refine the developing neural circuitry. Such processes affecting the brain's connectivity (mainly during early life) and functions constitute neuroplasticity, and are orchestrated by inputs from the environment and the resulting activity in the brain. Neuroplasticity firstly includes pruning, which only keeps useful and relevant neurons or axonal projections from a superset of circuits [23]. In addition, new individual synapses continuously form in a random way, and are stabilized or destabilized by ongoing neuronal activity of the pre and post-synaptic neurons[24, 25]. Since synapses are not binary variables, their individual strength can also be tuned in an activity dependent fashion. However, as we will see later, a layer of homeostatic mechanisms makes it so that such changes in synaptic strength are often distributed in the whole network and therefore hard to monitor. All those mechanisms are thought to be the cellular correlates of memory and learning, and will be discussed in more details in the last chapter of this thesis.

Since the dawn of modern neuroscience, a lot of efforts has been made to determine the rules underlying learning, not only for fundamental understanding of nature, but also to be able to exploit them into artificial systems. Several good candidates have been proposed and at least partially validated experimentally [26], including the one hypothesized by Donald Hebb [27] : *neurons which fire together wire together*. We will see in the next parts how such hypotheses have inspired the development of neural network-based artificial intelligence, and whether some of the concepts that have emerged from this field can be applied back onto biological neuronal devices.

Artificial neural networks (ANN)

A plethora of in silico ANN types were developed over the last 50 years, with as many different architectures and neuron models as there are possible applications to them : weather prediction, missile guidance, face recognition, automatic speech translation, etc... ANN organization usually involves an input layer, on which is clamped the data, and an output layer, where the actual output is simply read as neuronal activation. One of the first model of ANN, the perceptron, was developed by Rosenblatt [28] in the late 1950's so as to perform classification tasks. Inputs are given to the network, for instance, the weight, the size, the picture, etc... of an animal, leading to the activation of the appropriate neurons, in this scenario the one with the appropriate animal label, in the output layer (Fig. 7 A). The learning strategy is supervised : the weight of the connections causing the errors are changed until the output is the one expected. In practice, if for a particular input vector, an output unit is activated while it should not, strong connections between the active input units and this output unit are weakened. Reversely, if an output unit is not active while it should, weak connections between them are strengthen. After



many iterations, the perceptron builds in its weights an internal representation of the data allowing to discriminate the different inputs.

FIGURE 7. – **Perceptron : architecture and learning.** A- Architecture of a perceptron after it has learned to recognize cats from dogs based on two input values. Green arrows are excitatory connections and red arrows are inhibitory. The thikness of each arrow represents its weight. B- Diagram showing the perceptron updating one of its linear boundary as more training examples are added. (Credit for B : Elizabeth Goodspeed)

However it became rapidly evident that the classification capabilities of the perceptron were limited to linearly separable functions (Fig. 7 B) for instance a perceptron with two inputs and one output neuron can not learn the XOR function (where the output is turned on by activation of either input, but switched off when both inputs are active). The addition of *hidden* layers between the input and output layers was demonstrated to extend the complexity of the input-output function to non linearly separable functions, but such multilayered networks couldn't be trained effectively until algorithms to back-propagate the error inside the hidden units were found in the 1980's [29]. For 20 years, supervised learning of multilayered networks using error back-propagation algorithms remained the most popular method in the field of ANNs. The main hurdles were the huge training time, and the inconsistency of results due to the possibility of getting stuck in local minima.

In the early 2000's, a combination of biologically inspired hebbian rules were implemented in an algorithm allowing networks to learn efficiently, and in an unsupervised way, how to reconstruct the presented input data [30, 31]. By doing so, neurons incidentally learned to detect the most important features structuring the data, before the network is (possibly) fine-tuned with back-propagation in a supervised manner. Most of the training is therefore done by feeding the network with easily produced unlabeled data, and more layers can be added to achieve more and more subtle classification within the ever growing database. This breakthrough led to unprecedented classification capabilities, and inaugurated the era of deep learning from big data. If the

presented data are images, neurons in the first hidden layer typically learn to detect edges at different positions and with different orientations (Fig. 8) because all objects in our world have clear limitations in space. In deeper layers however, some neurons can become activated by pictures of very specific objects or even abstract concepts like *friendship* [32, 33, 34]. The receptive fields learned by neurons in deep neural networks match remarkably well with those found in the visual cortex of primates, confirming the relevance of hebbian plasticity rules. For sure, the discovery of other rules, at play in biological systems, will continue to foster the field of AI in the future.



FIGURE 8. – **Receptive fields inside a deep neuronal network trained on natural images from [32].** From left to right : receptive fields found in the first, third and fifth layer of a deep neural network, showing increasingly higher-level feature detection.

Those ANN usually refer to algorithms implemented on conventional computers. However, doing so presents a number of disadvantages. Firstly the time to process inputs on a simulated network increases quadratically with the number of neurons. Secondly, conventional computers are not energy efficient. The computers running the Blue Brain Project require for instance as much electricity as a small town while the human brain uses the equivalent of a 20W light bulb. Finally computers are known to be quite susceptible in terms of operating conditions, which might prevent the use of simulated ANN in extreme environments (in spatial probes for instance).

After the recent advances in ANN, different research teams [35, 36, 37] and companies (IBM, HP, Qualcomm) have started to develop special hardware allowing to run ANN faster, more efficiently and more robustly. The core of such neuromorphic computers usually consist in actual networks of electronic neurons, pre-connected with tunable weights; like in biological neural networks, the hardware and the software are indistinguishable from one another.

However, silicon chips are not the only possible option for neuromorphic processors. Real neurons constitute naturally an attractive material to implement neural networks and the learning algorithms originally drawn from them. As recent technological developments have made the manipulation of biological neurons and the reconstruction of networks more straightforward, we can rightfully wonder if discoveries made in the field of AI would work in biological implementations.

Biological neuronal devices : state of the art

Leaving well-behaved models and semi-conductors for the realm of biology is easier said than done. Using living materials imposes a lot of constraints to the experimenter who wishes to build something out of them. As everything that is alive can die, the first challenge is to keep said living materials healthy as long as necessary. Providing a physiological environment with the appropriate nutrients is of course the main determinant for survival. However, like they do *in vivo*, cultured neurons are subjected to aging, giving as a consequence an additional layer of temporal constraints. The development and maturation of cultured neuronal networks actually involves a large panel of processes through which neurons first grow and establish synapses with other neurons, then express different sets of genes as time goes, causing some major shifts in their electrophysiological properties. For instance GABA receptors gradually switch from excitatory to inhibitory during the first ten days *in vitro* [38] while NMDA receptors, involved in synaptic plasticity, are expressed at even later stages [39].

Provided that the basic needs of neurons are satisfied, and that they are in the right developmental stage, there is another hurdle : homeostasis. Cells that we grow *in vitro* have originally evolved different compensatory mechanisms to maintain an internal state that guarantees proper operation across a wide range of environmental conditions, for the sake of their common good, that is the survival of the animals they normally constitute. The cells will continue to fiercely regulate themselves when grown in a Petri dish, whether or not the functional implications are relevant to the experimenter. The sad corollary is that properties of interest that are incidentally connected to these homeostatic mechanisms can hardly be controlled experimentally, at least under physiological conditions.

Homeostasis postulates for instance the existence of an optimal average firing rate for neurons, determined as a trade-off between energy consumption and information processing efficiency, that neurons actively maintain by tuning either their synaptic inputs [40] or their intrinsic excitability [41]. For instance activity deprived cultures reinforce excitatory synapses[42] and weaken inhibitory synapses[43], making them more excitable. Conversely, over excitation of cultures induce changes that reduce the excitability of the underlying networks. This kind of network homeostasis has generally been linked to up or down-regulation of synaptic receptors [44]. Finding ways to control the states of living neural networks without affecting such internally regulated properties remains to this day the biggest challenge in building living neuronal computers. In spite of the complexity of the task, several groups have tried (and succeeded, to some extent) to use cultured neurons to perform computations. Surprisingly, few of them -if any- have based their work on ideas that did give promising results in the field of AI and ANN.

The vast majority of the work done to harness the computational power of neurons involved random networks grown on micro electrodes arrays (MEAs, Fig. 9). The electrical activity of neurons sitting on top of each electrode can be measured and translated ultimately into spikes. Reversely, it is possible to electrically stimulate neurons through the electrodes, the general idea being that the neuronal culture computes a function, taking as an input a spatiotemporal stimulation pattern, and returning as an output spike patterns on the different electrodes.



FIGURE 9. – **MEA culture dish and close-up look on the electrodes.** A weekold culture of $\approx 50\ 000$ neurons and glia from embryonic rat cortex, growing in a MEA and forming a dense network 1-2 mm across. Fifty-nine 30 μ m electrodes spaced at 200 μ m intervals connect a few hundred of the network's neurons to the outside world, by allowing their activity to be extracellularly recorded or evoked by electrical stimulation. Reproduced from [45]

Coding strategies were developed to control robots and animats [46, 45, 47], by converting sensory inputs (position in the environment, presence of an obstacle, etc...) into stimulation patterns, and the neural responses into actions (displacement, turn, etc...). Since it is neither possible to control which neurons sit on each electrode, nor how they precisely connect together, the coding strategy to obtain initially appropriate input-output function is not only application specific, but also culture specific. Another drawback is that a lot of computation is done outside the neural network (*i.e.* in a conventional computer) in order to decode the spike trains and encode the stimulations, undermining the role of actual neuronal computation.

Once the coding strategy is fixed however, modifications in the inputoutput function are entirely attributable to changes in the network's connectivity, usually interpreted as a manifestation of synaptic plasticity. It would be therefore possible to reprogram, to some extent, such neuronal processor while keeping the same coding strategy. Experimental and theoretical work describe different methods [48, 49, 50] to change the network's connectivity in a goal-oriented manner using synaptic plasticity, but also reveal some limitations. For instance, learning a new function generally leads to the modification of preexisting ones. This can be attributed to the fact that the different neuronal sets implementing those functions overlap to such extent that it is difficult to only affect one at a time. An other problem is that networks growing onto the electrodes spontaneously establish preferential communication pathways which are difficult to overwrite and therefore greatly reduce the scope of learning.

Being able to control the topology and connectivity of cultures seems therefore to be a prerequisite for developing consistent coding strategies and efficient learning *in vitro*. Since *in vivo*, the initial coarse-grained connectivity emerges from an interplay between intrinsic growth processes and physical constraints, we could apply the same principle to control the connectivity of neuronal cultures. While the former are hardly accessible experimentally (as they are genetically encoded), a number of micro-fabrication technologies have enabled us to control the microenvironment of neurons with high precision and reproducibility.

Instinctively, one would think of building a neural network by manipulating individual neurons. Micro-patterning techniques (Fig. 10) have indeed become sophisticated enough to spatially confine single neurons accurately with adhesion spots [51], and even orientate neurites outgrowth and differentiation [52]. However populating the patterns in a reproducible way remains problematic. As a cell suspension is applied on the substrate, the different slots are filled randomly, leaving some of them empty or wrongly populated. The probability of filling properly a network by chance decreases exponentially with its size, which limits in practice single neuron networks to very small sizes until efficient cell capture technologies exist.

Instead, many approches have focused on shaping the networks at larger scales, organizing the connectivity between groups of neurons instead of individual neurons. This can of course be done using micro-patterning techniques with larger motifs. Alternatively, organization of neurons at this scale can be controlled by direct physical confinement. This is generally achieved using micro-fluidics chips. They consist in molded silicon polymer (polydimethyl-siloxane or PDMS) covalently attached to glass coverslips through plasma-activated bonding. The patterns originally engraved in the PDMS surface are sealed by the glass, creating micro-channels that can be filled with adhesion molecules and later seeded with cell suspensions. The advantages of cultivating neurons in micro-fluidic devices are numerous [53, 54]. Most notably the chips are easy to assemble and yield highly reproducible culture conditions, which is probably the reason why this technology prevails in commercial solutions aiming at *in vitro* network reconstruction.

If the activity of single neurons can still be exploited in population level networks (using patterns on MEAs for instance [55, 56, 57]), the coarse-grained activity of populations can be more conveniently used to code and process information. In the most extreme case, the network is treated as a continuous



FIGURE 10. - Principle of two micro-patterning techniques. A- Lift-off photolithography: A cytophobic substrate is coated with a thin layer of photoresist and illuminated through a photomask endowed with the desired patterns. Depending on the type of photoresist, the illuminated or non-illuminated areas become dissolvable, eventually exposing the underlying substrate to subsequent treatments while leaving other areas protected by the photoresist layer. Adhesive molecules, such as poly-lysine are applied onto the substrate, and are allowed to adsorb everywhere. The sacrificial stencil is thereafter lifted-off, leaving patterns of adhesive molecules on a cell-repellent background. One major drawback of lift-off technology is that the fabrication is time-consuming and requires elaborate clean-room equipment. B- Stamping: The stamps are usually molded with a soft silicon polymer, polydimethylsiloxane (PDMS), from a master mold. Since many stamps can be made from a single mold, and since each stamp can be used several times, clean-room operations are reduced tremendously. The main drawback is the inconsistency of patterns density due to inhomogeneous coating of the stamps and inhomogeneous transfer of the adhesive molecule to the substrate.

excitable mesh where topographical boundaries define the flow of information inside it. The group of our collaborator Elisha Moses in particular, showed that it was possible to implement robustly different simple functions [58], including AND gates, diodes and oscillators, by growing the neurons on patterned substrates as a mean of controlling the bulk connectivity of the network. As the operations performed by such devices only depend on their coarse-grained connectivity, they can be fabricated in a reproducible manner. Furthermore the read-out, achieved through activity-dependent fluorescent dyes (see Chapter III for details), is very robust as it is done on areas integrating the activity of many neurons at once. However, the resulting neuronal devices are not *trained* to perform those computations. The required connectivity emerges from the interaction of spontaneous neuronal growth with the geometrically constrained substrate, not from experience. The programming of the device is therefore done beforehand in the substrate, and its purpose can not be changed afterwards.

Here I propose a new paradigm for neuronal devices. Firstly I use microwells to compartmentalize cultures into neuronal populations, individually acting as super-neurons. These super-neurons are both robust and customizable as their properties emerge from the mean composition and density of the seeded cell suspension, which can be controlled accurately. They are also easy to read, as neuronal populations can display all-or-none bursts of activity which are easily detectable. Even if the amplitude of such bursts can vary and therefore carry information relevant to neuronal computation [58], they can also be seen as stereotypical events corresponding to super-neuron spikes; the investigation of temporal codes is therefore straightforward in such scheme.



FIGURE 11. – **Minimal neuronal devices.** Top : Two populations of \approx 700 fluorescent neurons are connected through micro-tunnels, the geometry of which can be controlled to yield unidirectional connectivity. Only a fraction of neurons are fluorescent here. Bottom : Three populations device (two inputs and one output) showing neurons stained for the cytoskeletal protein MAP2. Two and three populations devices constitute the building blocks of more elaborate devices.

Secondly, the populations are connected through arrays of axon-selective micro-channels which control topographically the initial connectivity between them. Since the directed connections in the network are spatially separated, each can be reinforced individually without affecting the others. This type of architecture can bypass the limitations of previous approaches where learning is hindered by highly reciprocal and overlapping connections in the culture. Additionally, network topologies developed in the field of ANNs can be easily implemented using this approach (Fig. 11). The fact that the network topology inside each population is not constrained in such devices means that a lot of computational power is potentially lost. In the worst case scenario, and provided that populations can actually act as super-neurons, the number of useful computing units would be the number of populations, which is far less than the actual number of neurons. However, random sub-circuits inside each population could be exploited to multiplex different functions on each unit, as it is done on unconstrained cultures growing onto MEA. The computational capabilities of population units are therefore difficult to predict, but they would at least support the properties of the units inside a perceptron. In this thesis, I present new tools necessary for the design, fabrication and characterization of population based neuronal devices. The first chapter will present a model allowing to predict axonal growth efficiently inside PDMS microchips to control the connectivity between the different populations. The second chapter describes a theoretical model known as quorum percolation which links super-neurons behaviors with experimentally accessible parameters (density and composition). In the third chapter I will present methods and instrumentation to interface neuronal devices. Finally, results regarding computation, plasticity and learning will be presented in the last chapter.

Controlling connectivity in neuronal devices

Introduction

In vitro reconstruction of neural circuits represents a interesting approach for the investigation of cognitive functions, developmental mechanisms and neurodegenerative disorders otherwise difficult to access in animal models. Microfluidics has emerged as an important technology in academic research [59, 60, 61] and even more remarkably in the industry (Xona microfluidics, Millipore, MicroBrain Biotech, Axol, etc...) to control the architecture of neural networks *in vitro*. Using microfluidic devices offers indeed many advantages over simple patterning technics, including high manufacturability, robustness, controllable environment, etc... [53, 54, 62].

The connectivity in neuronal chips is usually controlled using microtunnels that physically allow axons to grow between different populations of neurons while keeping the cell somas in their respective compartments. However, using such topographical guidance becomes rapidly challenging when networks get larger and more complex. Since the range of applications of such chips is ultimately limited by the connectivity between the neuronal compartments, the development of technologies allowing to control axonal growth reliably is critical for future research.

Some of our earliest attempts to operate axonal projections via PDMS microstructures (Fig. 12) have highlighted the difficulty to predict the growth of axons without prior knowledge of the elementary axon/substrate and axon/axon interactions underlying constrained axonal growth. In particular, axons tend to follow the walls inside micro-tunnels and micro-chambers, giving rise to unwanted connections. These can furthermore be amplified by the propensity of axons to follow each other and fasciculate into thick nerve-like bundles.

If one would instinctively want to avoid such behaviors, we aimed instead at exploiting them to control axonal growth. I developed therefore a predictive model recapitulating axonal growth under topographical constraints, accounting for the behavior of freely growing axons, interactions with the edges, and


FIGURE 12. – **A failed attempt at building an axon merger.** Axons (in green) coming from two input populations (populations not visible on the picture) converge appropriately into the central chamber, but instead of escaping the chamber towards the common exit (top), they mostly grow horizontally and establish unwanted connections between the input populations.

interactions with other axons. The various experimental observations necessary to tune the model were collected from time-lapse movies of mice cortical axons growing into dedicated microfluidic chips, containing batteries of standardized guides and obstacles.

I first present the results for the fit of the different ingredients of the model, namely how straight individual axons grow, their affinity for edges, and their fasciculation strength. I then investigated the importance of the different ingredients in various geometries previously tested experimentally. I show that the relative importance of each ingredient is geometry dependent. This shows there is no universal driving force behind the success or failure of a particular design and that only a model incorporating all the aspects of axonal growth could accurately predict the effectiveness of different designs. Such exhaustive model could further be implemented in automatic optimization algorithms for unknown geometries and permit the emergence of neuronal devices significantly more complex that the ones we currently use.

Methods

Model of axonal growth

The model was designed to simulate axons growing on an environment map by adding iteratively new segments in the appropriate direction. The deviation from previous growth direction is drawn at each time step from a probability density function defined on $[-\pi,\pi]$ which constitute the core of the model.

This density function is firstly determined by intrinsic growth parameters reflecting the characteristic persistence length of freely growing axons. It is however affected by an environmental modulator accounting for the accessibility of different areas in the pixel map (1 for accessible pixels, 0 for inaccessible pixels). Additionally, the edges and axons appear as special pixel values on the environment map : the pixels at the edges take the value ϵ (edge affinity constant), while pixels covered by axons take the value ϕ (fasciculation constant). Details about the intrinsic probability distribution, ϵ and ϕ are given later in this section.

The environmental modulator function is obtained by probing the substrate around the axon tip in all directions. For each direction, the value of the environmental modulator is the maximal pixel value along this direction on the environment map, and within a distance from the tip (or before, if an inaccessible area is met). The effective probability distribution is finally obtained by multiplying the intrinsic probability distribution with the environmental modulator and normalizing the result (Fig. 13).



FIGURE 13. – **Modeling axonal growth in heterogeneous substrate.** The directions of the next axon segment (one of the many possible dashed purple arrows) is drawn from an effective probability distribution that takes into account the affinity of axons for edges and other axons.

Obstacle arrays design

The chips consisted in macro-channels (1 mm wide and 15 mm long) in which neurons were seeded. The macro-channels were connected laterally to small chambers (80 μ m wide) via individual axon channels. The chambers, containing different topographic obstacles, were open on the other side towards a medium reservoir, so as to allow diffusion of nutrients (Fig. 14 A).

Different types of obstacles were used. Rectangular and triangular obstacles, positioned on the paths of axons were used to characterize their behaviors as they met PDMS walls at various incidence angles. Additional designs were used to characterize the behaviors of axons at corners of various angles (called α), where they can either grow straight, or follow the micro-structures edges.



FIGURE 14. – **Design of the chips.** Diagram showing the organisation of the chips as well as the different geometrical constraints.

Micro-fabrication

The master molds for the PDMS chips were made by exposing spin-coated layers of SU-8 photoresist on a silicon wafer. The axonal structures (axon channels and obstacles) were imprinted in a SU-8 2005 layer with a height of 5 μ m, so as to exclude larger cell bodies. For the somato-dendritic channels, a layer of SU-8 3050 100 μ m high was used (the 2000 series gave too poor contrast for alignment). Each layer was soft baked, aligned with its photomask (for the second layer) using a mask aligner, exposed, post-exposure baked and developed in SU-8 developer following the guidelines in the SU-8 manuals.

PDMS base and curing agent (10 :1 ratio) were mixed and degassed under vacuum before the mixture was poured onto the master molds. After 1 hours in a 70°C oven, PDMS parts were turned out from the molds. Holes were punched at the extremities of the macro-channels and reservoirs. Clean 32 mm coverslips and PDMS parts were treated 1 min and 30 sec in air plasma respectively, and sealed together. The covalent binding between the plasma activated surfaces was accelerated by putting the chips 3 min on a 70°C hotplate.

Immediately after, while the inside of the chips was still hydrophilic, poly-D-lysin solution (0.1% m/v ratio) was injected in the macro channels and allowed to penetrate through the axon channels into the micro-chambers. Poly-D-lysine was incubated for at least two hours to allow its homogeneous adsorption inside the chips, then rinsed twice with PBS. In order to image multiple chips at once, 6-well plates were perforated, and the chips were glued to the bottom using PDMS.

Cell culture

Primary neurons were obtained from P0 (new born) GFP mices kindly given by the group of Edith Heard (Génétique et biologie du développement, Institut Curie). The pups were decapitated and their brains were micro-dissected on ice in L-15 medium without phenol red (Life technologies) supplemented with 0.6% glucose and 0.2% gentamicin. Cortices were digested in papain solution (papain 100 units, DNAseI 1000 units, L-Cystein 2 mg, NaOH 1M 15 μ L, EDTA 50 mM 100 μ L, CaCl₂ 100 mM 10 μ L, dissection solution 10 mL) at 37 °C for 20 minutes. After digestion, the supernatant was carefully removed and replaced with 10 mL of plating medium (MEM without glutamine supplemented with 0.6% glucose, 1% GlutaMAX, 5% Horse Serum, 5% Fetal Calf Serum and 0.1% B27) supplemented with 25 mg of Bovine Serum Albumine for 5 min to block the papain. Supernatant was removed, replaced with plating medium and tissues were triturated with a micropipette. Neurons were counted on a Malassez cell in 50% trypan blue, centrifuged 6 min at 100g, and resuspended at 20 000 cells per microliter. The suspension was injected into the macro-channels inlets (3 μ L per channel), yielding a density of 2000 neurons/mm2. The seeded chips were incubated half an hour in a humidified, 37°C and 5% CO₂ incubator to allow the attachment of neurons to the Poly-D-Lysine substrate. Each chip was finally covered by 2 ml of serum-free medium (Neurobasal, B27 4%, GlutaMAX 1% and FCS 1%) and the cultures were placed back into the incubator. The time-lapse experiments were initiated after 2-3 DIV.

Timelapse acquisition

The culture medium in each well was replaced by external medium (NaCl 129 mM, KCl 4 mM, MgCl₂ 1 mM, CaCl₂ 2 mM, Glucose 10 mM, HEPES 10 mM, pH 7.4, B27 2%, gentamicin). This external medium allowed the survival of

neurons for several days outside the incubator without temperature or CO_2 control. A special glass cover for the 6-well plates was used instead of the original plastic covers, in order not to alter DIC observations. The cover was also equipped with heating resistors to prevent condensations above each chip. After replacing the cover, the plates were sealed with parafilm to avoid media evaporation during the experiment, which appeared to be the most critical factor for survival.

The timelapses were done on an inverted microscope (Nikon Ti-E) with motorized stage, shutters, and focus systems, allowing complete automation over several days. A long working distance 20× objective was used.

Because of the large number of fields to be imaged, the different positions were computed by linear interpolation of 4 reference points manually determined using NIS (Nikon Imaging Software). The reference points were fed to a MATLAB script to compute automatically the positions of 44 different fields covering the 124 different obstacles in each chip. The output, an xml file, was finally uploaded back to NIS.

A picture of each field was taken using differential interference contrast (DIC) and GFP fluorescence channels every 30 minutes for one week.

Pre-processing

The images at each time point, channel, and field of view were corrected for rotation, and the positions of the different chambers were determined by template matching on the DIC channel using home made algorithms (MATLAB). The regions of interest were extracted as individual TIFF files, each containing a chamber (Fig. 15).

Extraction of intrinsic growth parameters

In order to isolate intrinsic growth processes from interactions with obstacles and other axons, the pool of data was screened for the fields where the first axon did not interact with the wall surrounding the entrance and continued straight. A picture was extracted of the selected time series at the latest time step before the first axon has met an obstacle or before a second axon has arrived. At this timescale, axons are quite representative of the actual growth path, contrarily to older axons that tend to straighten because of tensile forces. Using a custom tracing interface (MATLAB) the axons in this final set of pictures were traced with 10 pixels (or 3 μ m) long segments, which is inferior to the typical persistence length of axons and should therefore capture the growth process accurately. Angles between successive segments (Fig. 16) were computed over the different axons and we obtained a gaussian like distribution of angles. In this ballistic regime, it is possible to normalize the measured changes of orientation between two consecutive segments relatively to the the



FIGURE 15. – **Pre-processing of images.** Top : Raw picture taken in DIC microscopy, showing the actual obstacle chambers inside the microfluidic chips. Bottom : Results after the pre-processing steps showing different extracted patches.

length *d* of the segments. The normalization factor is $\sqrt{\frac{d}{3} + \frac{1}{2} + \frac{1}{6d}}$ (demonstration in the appendixes). The normalized angle distribution was used to fit the gaussian distribution used in the simulations. The fit was done using a genetic algorithm.

Extraction of the edge affinity constant

In order to quantify the extend to which axons prefer to grow along the edges of PDMS structures, I used experimental data collected from sector shaped obstacles. I did simulations of axons growing in similar geometries with different parameters : the sector angle α and the edge affinity constant



FIGURE 16. – **Unconstrained axonal growth.** Diagram showing how an initial axon segment (in purple) would behave differently (red or blue dashed segments) depending on the angle distribution between successive segments.

 ϵ . The number of axons growing along the edge 10 μ m before and after the turn (Fig. 17) was measured in each simulation. For each ϵ , the angle at which the ratio $\frac{after}{before}$ dropped below 0.5 (the critical angle) was determined. This allowed us to determine a range of values for ϵ yielding critical angle values in agreement with those estimated experimentally.



FIGURE 17. – **Edge affinity.** Diagram showing how an initial axon segment (in purple) would behave differently (red or blue dashed segments) depending on the edge affinity constant. The angle of the corner is showed in red. The two regions before and after the corner are displayed in pink and orange circles respectively.

Extraction of the fasciculation constant

Since the micro-channels leading to the chambers are thin and straight, axons entering the chambers are initially all in contact and growing in the same direction. While some of them go separate ways, other keep following each other (Fig. 18). I extracted the traces of the first and second axons growing inside chambers and evaluated the fraction of axons pairs maintaining contact over a distance of 20 micrometers or more (i.e. where the terminal distance between the two axons was smaller than 1 μ m). By running simulations of axons pairs under the same initial conditions and with different ϕ values, we could tune the axonal affinity constant to reproduce the experimental results.



FIGURE 18. – **Axon fasciculation.** Diagram showing how the initial axon segment (in purple) of the second incoming axon would behave differently (red or blue dashed segments) depending on the strength of fasciculation.

Results and discussion

Fitting the model to experimental data

The distribution of normalized direction changes (Fig. 19) was best fitted with a normal distribution a standard deviation $\sigma \approx 8,2^\circ$. Trying to fit instead a combination of 2 normal distributions led to either the same standard deviation for the two components, or to a component whose amplitude was negligible before the other, indicating the directionality distribution is actually better defined by a single normal component.

After fixing intrinsic growth parameters, we could focus on modeling the interactions between axons and the edges of microstructures, a phenomenon observed in our experiments. We observe in particular that axons can actually keep following edges around corners whose angles are larger than a critical value (Fig. 20 A). From the experimental data collected for that matter, we could derive a gross estimate for the critical angle (between 100° and 150°). In the simulations, we find that in this range, the critical angle is quite robust against the edge affinity constant. Given the uncertainty on the estimated critical angle, we obtained an even broader range of plausible edge affinity



FIGURE 19. – **Unconstrained axonal growth.** A- Experimental distribution of the normalized direction change between consecutive segments extracted from freely growing axons. B- Traces of the axons used for the distribution in A (n=105 axons). C- Simulation of freely growing axons where the deviation between consecutive segments are drawn from a normal distribution fitted to the distribution in A (n=105 axons).

values. An edge affinity $\epsilon = 10^2$, corresponding to a critical angle of 135° was chosen as a representative value in our simulations.



FIGURE 20. – **Edge affinity and critical escape angle.** A- Behavior of axons growing around corners. The corner angle is represented in red. Beyond the critical angle (here between 154° and 96°), the vast majority of axons stop following the edge when they reach the corner. B- Simulation results indicating the fraction of axons that keep following the edge after the corner, as a function of the corner angle and the relative affinity of axons for edges. Fractions of 0.5 are indicated by the red curve. The green double arrow represents the range of acceptable values for the critical angle. The blue arrow represents partially the corresponding ϵ values

Lastly, the axonal fasciculation constant was determined from our experimental results. Over 30 observed axons pairs initially in contact, 66.7±8.6%, did maintain contact over a distance of 20 μ m or more. Using simulations I calculated the equivalent probability for a wide range of fasciculation strength (Fig. 21). I could in this way determine the relative fasciculation constant to lie between 10² and 10³.



FIGURE 21. – Fasciculated fraction after a growth of 20 μ m as a function of the fasciculation constant. Each point was obtained from 1000 axons pairs. The standard deviation is shown in black vertical bars.

A summary of the different parameters values chosen for simulations is shown in Table 1.

Parameter	Standard growth deviation (σ)	Edge affinity (ϵ)	Fasciculation (ϕ)
Value	8,2°	100	200

TABLE 1. – Parameters for axonal growth simulation.

Contrarily to other biophysical models found in the literature, ours is purely phenomenological; its goal is to predict axonal growth, not to explain the underlying mechanisms. Although it contains the main elements shaping, in our opinion, the way axons grow inside micro-fluidic devices, there is a number of points about the way they are implemented and articulated together that should be taken into considerations.

Firstly I did not include in the model correlations in the growth direction over more than two consecutive time steps. For instance an axone could grow by doing short scale undulations while maintaining a straight course at a larger scale. Taking into account correlations over greater distances could at least correct for possible artifacts obtained during axon tracing (for instance zigzags around a straight axon). Secondly, it is difficult to determine if the single normal distribution of turns that I used, which converges very quickly to 0 away from the growth direction, can faithfully describe the distribution of sharp turns as we lack statistical data on these rare, yet very important events. This quickly decaying distribution is also problematic to compute the effective growth direction distribution, for the environmental distribution is multiplied by almost 0 far from the ongoing growth direction. Although it can be compensated by using very large values for edge affinity and fasciculation constants, there might be more clever way to compute the effective growth direction distribution than by simple multiplication. Finally it is important to mention that the values for the different model parameters are most certainly dependent on the type and age of neurons growing in the chips, as well as the micro-fabrications methods. However, as demonstrated in this part, fitting such model is rather straightforward when using the appropriate battery of experimental conditions.

Predictive power of the model

The predictive power of the model was tested by simulating axonal growth under different geometries that were also assessed experimentally.

Test 1 :

The first geometry consisted in rectangular obstacles placed on the axon path in different orientations. Experimentally, we observe that the fraction of axon passing the obstacle through the left or through the right depends on the orientation of the obstacles (Fig. 22 A). In order to quantify this splitting, I integrated the fluorescence on either the left or right side of the chamber beyond the obstacle, and used the relative fluorescence of the left side as an indicator of the asymmetric splitting. Plotting this indicator as a function of the obstacle orientation yielded a very characteristic function.

Simulations were done in the exact same geometrical constraint and using the same scale (3 pixels/ μ m) as in experiments. The results of simulations, exported as pictures, could therefore be treated in the exact same way than the experimental data. Using the set of parameters previously selected (step=1,5 μ m, σ =8,2°, ϵ =100 and ϕ =200), I obtained results quantitatively similar to experiments. In order to determine if those results are really working because of our models parameters, I did additional simulations with different persistence length, edge affinity constant, and fasciculation constant.

A very strong determinant of the splitting profile appeared to be the persistence length of the growing axons. It is clear that the asymmetric splitter is more efficient when axons grow straighter (Fig. 23 A-B).

Edge affinity was in this geometry not critical in the splitting mechanism as its suppression did not strongly altered the splitting profile (Fig. 23 C). We can explain this by the fact that whether the edge affinity ϵ is 0 or 100, the critical angle is significantly larger than 90° and most of the axons enter the chamber straight to the obstacle. However a very strong edge affinity $\epsilon = 10000$ reduces the strength of the asymmetric splitting (Fig. 23 D), because more axons follow the walls immediately when they enter the chamber. Since they have at this



FIGURE 22. – **Asymmetric splitting of axon bundles.** A- In green, median fluorescence distribution (n=10) of axons interacting with a rectangular obstacle at a 50° angle after 2 weeks of growth (those pictures were not used for tracing). The fluorescence of axons bypassing the obstacle through the left and through the right was integrated in the magenta and yellow rectangles, respectively. B-Relative fluorescence on the left side as a function of the obstacle angle θ . Blue points are data points from a single experiment with 31 different orientations. In red are values extracted from images of the same dataset flipped horizontally, showing the expected symmetry. C- Example of image obtained from simulations, showing the path of axons. The appearance of axons vary from experimental data presented in A because of tensile forces that straighten the axons in older cultures. This straightening is however not expected to affect fluorescence measurement. D- Average profile extracted from simulations (σ =8,2°, ϵ =100 and ϕ =200, n=10 simulations of 30 axons) with the same procedures as in A and B.

point equal chances to turn left or right, a big part of the incoming axon beam is split symmetrically.

Finally, the average profile was not strongly affected by dropping the fasciculation constant. However its value is important to account for the variability between individual experiments (Fig. 23 E-F). As more axons grow in the chamber, fasciculation tends to amplify the axonal distribution already present in the chamber instead of converging to the same mean distribution we would get from independently growing axons.

Test 2 :

I tested in addition a new geometry of axon diodes in collaboration with Jean-Baptiste Durand (Institut Curie). These diodes are based on special junctions that exploit the principle of critical release angle to affect axonal growth differently in one or the other growth direction (Fig. 24 A). After performing



FIGURE 23. – The importance of the different ingredients of the model. Average profiles extracted from simulations (σ =8,2°, ϵ =100 and ϕ =200, n=10 simulations of 30 axons) except for A : σ =16.4° B : σ =4.1° C : ϵ =0 D : ϵ =10000. E and F : Results obtained from n=5 independent simulations of 20 axons without (E) or with (F) fasciculation. The mean is displayed as a black curve and the standard deviation is shown as black vertical bars.

fluorescence quantification on either side of these junctions, this system appears to yield experimentally a selectivity for the forward direction of ≈ 5 (*i.e.* 5) times more axons grow pass the junction in the forward direction compared to the reverse direction). Simulations under control parameters gave once again quantitatively similar results (5,88±1,00). As before, I changed the model parameters to investigate the mechanism yielding the direction selectivity of the diodes (Fig. 24 C). The efficiency of this device was mostly dependent on the edge affinity constant, as its suppression highly reduced the filtering power of the diodes, although surprisingly, not completely $(2,63\pm0,57)$. How straight the axons grow also affects the efficiency of the diodes. As expected, axons that grow straight have the tendency to ignore lateral connections, and the direction selectivity is therefore lowered (2,62±0,23 for axons growing twice as straight as in control parameters). Finally we expected the fasciculation to reduce the direction selectivity, as the few axons that initially manage to take the wrong path open new routes for other axons to grow onto. However simulations show that this is not the case. Even if the "leaks" can be amplified by fasciculation, we can understand that the first axons to grow are likely to



follow the desired path and increase the chance for upcoming axons to behave properly. More experimental and numerical work is required to assess the effect of fasciculation when axons grow from both directions at the same time.



Conclusion

In this part we have examined axonal growth with the engineer's eye. Our model is not inspired by biological growth mechanisms and has therefore little explanatory power, however I have demonstrated that such simple model can already account for many experimental results. While there is room for improvement in the model, we can easily imagine how simulations can help in the design of neuronal chips by saving the time and costs associated with an experimental trial-error routine (Fig. 25).

Moreover, as in simulations we can affect individually the different components shaping axonal growth, it is possible to identify the driving forces behind



FIGURE 25. – *In-silico* optimization of junctions. Simulations show that the effectiveness of junctions such as the one presented in Fig. 12 is extremely sensitive to the precise positioning of pillars, in a manner which is difficult to predict intuitively.

inefficient designs and develop specific strategies to tame them. Alternatively, a user without any prior knowledge on axon guidance could still use this model, by only specifying the desired connectivity in a symbolic form, and letting an optimization algorithm come up with the most efficient design. Such tool can therefore help neuronal device to come into the light.

Neuronal populations as computing units : theoretical modeling

Introduction

Building neuronal devices based on populations instead of single neurons networks presents numerous practical advantages regarding micro-fabrication, but also finds its ground in their interesting functional properties.

One of the most important functional feature of cultured neuronal networks is their ability to produce bursts, that are global and acute increases in neuronal activity. The general mechanism through which bursts build-up in a population of neurons is fairly simple : each time an individual neuron fires (spontaneously or through a stimulus) it excites synaptic neighbors, increasing in turn their chance of firing. Because of this positive feedback, an avalanche that spreads quickly to the entire network can be initiated when a critical fraction of active neurons is reached. Typical bursts of activity last only for a few tens of milliseconds; burst termination is mainly due the depletion of neurotransmitter vesicles in synapses[63] but can also be due in some cases to the increase of inhibitory feedbacks, which reduce the overall excitability of the network. Those processes also impose a bursting refractory period to neural populations.

There are several theoretical and practical reasons to be interested in using bursts to code information in population networks. Firstly, we can draw interesting parallels between bursts in populations of neurons and action potentials in individual neurons : they both are all-or-none events distributed at discrete times, present a threshold-like activation function, and have a well define temporal profile ending with a refractory period. All the codes previously discussed for spikes can therefore be investigated in networks of bursting populations. Secondly, bursts are clearly standing out from the background activity, which allows unambiguous reading of populations states. Finally, bursts seem to be a very resilient property of neural networks in general ; they were observed across cultures of neurons from various origins, and under different experimental conditions. This makes bursts an ideal target for developing robust and universal coding strategies for neuronal devices.

If bursts are qualitatively similar in all cultured networks, we can observe quantitative differences in their amplitudes, durations, rates, etc... depending on experimental conditions. Understanding how bursts parameters depend on experimental variables is therefore critical to build consistent and reproducible devices. Theoretical models have been very helpful to this purpose.

Among these, the quorum percolation (QP) model offers a simple and general mathematical framework to explain recruitment of neurons in a burst. Neuronal populations are represented by a set of nodes (the neurons) that are connected through directed links (the synapses). Nodes can be in two different states, active or inactive. A node becomes active either if it is externally set as active, or if at least a quorum m of its inputs are active themselves. This process is recapitulated by a single mean-field self-consistent equation :

$$\phi = f + (1 - f) \sum_{k=m}^{\infty} p_k \sum_{i=m}^{k} \binom{k}{i} \phi^i (1 - \phi)^{k-i}$$

where f is the fraction of neurons initially set as active, ϕ is the final fraction of neurons activated after propagation, p_k is the probability of a neuron to have k incoming neighbors and m is the quorum. This equation reads as follow : the neurons which are active at the end of the cascade (ϕ) are either the neurons initially set as active (f), or, among those which were not directly activated (1-f), those that have enough active neighbors to be active ($\sum_{i=m}^{k} {k \choose i} \phi^i (1-\phi)^{k-i}$ being the probability that a neuron with k incoming links has a least m active neighbors).

This equation is remarkable in many ways. Firstly, the predicted behavior of populations is in excellent agreement with experimental data[64]. Secondly it links in a transparent way the connectivity of the networks (p_k distribution) and the excitability of single neurons (m), which are experimentally controllable parameters (see discussion at the end of this chapter), with the activation function of the neuronal population (the relation between ϕ and f) which is ultimately what interests us for computation. In particular, beyond a critical m_c value for m, the model predicts that the network activation function loses its threshold-like property and becomes continuous (Fig. 26). Finally the model and the associated equation offer by their simplicity a fertile ground for the exploration of other biologically relevant ingredients (inhibitory neurons, decay, etc...).

In this chapter I present two papers which describe developments and variations around the QP model. The first paper extends the concept of quorum to continuous values, allowing a finer characterization of the link existing between activation functions (in particular the value of m_c) and the connectivity of the network. The second paper describes the effect of randomly decaying signals on the activation functions. It appeared that even infinitely small decay



FIGURE 26. – Activation functions for different quorum value. $\frac{\phi-f}{1-f}$ corresponds to the probability for a neuron that was not externally stimulated to be activated during the cascade. The distribution of incoming links in this case is a normal distribution of mean $\bar{k} = 50$ and standard deviation $\sigma = 10$. The discontinuous threshold-like activation functions become continuous for $m \ge m_c$ (here between 35 and 40).

disrupted the discontinuous (threshold-like) activation functions observed without decay. I discuss how this impacts the interpretation of previously obtained experimental results.

These two papers present very general results that could be useful in various scientific fields (physics, biology, sociology, ecology, etc...) because QP is relevant to many other phenomena like spread of rumors, epidemics, decision making, etc... However I will restrain the discussion more specifically on the implication of these theoretical results for our undertaking of building neuronal devices.

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Effective non-universality of the quorum percolation model on directed graphs with Gaussian in-degree



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HIGHLIGHTS

- Collective behavior of dissociated neuronal cultures.
- Continuous prolongation of the quorum percolation model.
- Thorough numerical calculations in the infinite limit behavior.
- Accurate thresholds and critical exponent associated with the percolation transition: non universal behavior.
- Relation between critical parameters and topological properties of the network.

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ABSTRACT

We investigate a model derived from bootstrap percolation on a directed random graph with Gaussian in-degree useful in describing the collective behavior of dissociated neuronal networks. By developing a continuous version of the model, we were able to provide accurate values of the critical thresholds and exponents associated with the occurrence of a giant cluster. As a main result, it turns out that the values of the exponents calculated over a numerical accessible range covering more than two orders of magnitude below the critical point exhibit a slight dependence upon the connectivity of the graph.

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Percolation models have been successfully applied in describing the transmission of information through lattices of sites and links [1,2]. In this respect, they are expected to provide a useful framework for signal propagation in neural networks. The first theoretical attempt in describing the emergence of a giant cluster experimentally observed in dissociated neuronal cultures in vitro was based upon a link percolation model on a graph [3]. Later, a more sophisticated model, the so-called quorum percolation model derived from the bootstrap percolation one was proposed [4,5]. In its original version, bootstrap percolation [6] is a percolation model on a lattice, where each basic element is a two-level system (active or inactive) located at a node; more recently, bootstrap percolation has been studied on random graphs [7]. The main additional feature that a model of neuronal network has to take into account is the fact that electric action potentials cannot propagate along axons in both directions. Hence the underlying graph of such a quorum percolation model must be directed. The connectivity of the graph is encoded in the adjacency matrix whose elements a_{ij} are equal to 1 if the nodes indexed by *i* and *j* are connected, 0 otherwise. Starting from a state of the network where a given fraction *f* of nodes is initially set active, information spreads through the network according to a threshold rule: a node becomes active if a fixed number *m* of its first neighbors are active. Although critical phenomena associated with directed percolation on one hand and bootstrap percolation on the other hand

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have been widely investigated, a thorough analysis of the critical phenomena associated with the quorum percolation model described above is not available, and has to be carried out specifically. Although bootstrap and quorum percolation models are close, the fact that links are directed rather than symmetrical is expected to impact on the critical behavior; moreover, the dissymmetry of links makes analytical approaches much more difficult, and very scarce results are available. An issue of fundamental importance in studying percolation model on graphs is the possibility to extract information related to the connectivity of the network from a critical behavior [3]. In this way, E. Moses and his coworkers were able to deduce from an experimental phase diagram that the degree distribution of the neuronal cultures they studied was a Gaussian one and to estimate the mean connectivity of the neuronal networks. It should be pointed out that the connectivity should not be understood here in the usual geometrical sense in a physical space, but from the point of view of the information provided by the adjacency matrix: such-and-such node are connected by a directed link, no matter where they are in the embedding physical space.

This paper is devoted to a thorough numerical study of the critical behavior occurring in the framework of the directed quorum percolation model in the thermodynamical limit.

1. Theoretical framework and numerical method

The network is made up of *N* nodes, where each of them is a two-level system: it can be either active or at rest. For each node in the network, *k* incoming links are randomly chosen among the N - 1 other nodes according to a probability p_k ; the implemented algorithm rules out backlinks (if $a_{ij} = 1$ then $a_{ji} = 0$). Hence, the connectivity of the directed graph \mathcal{G} is entirely determined by the degree distribution of incoming links per node. At t = 0, a fraction *f* of the nodes drawn randomly among the network is activated. The activation process of the network is described by a discrete time dynamics [5] with a step Δt during which each node integrates the signals sent by its incoming first neighbors [8]. A variable $V_i(t)$, assigned to each node *i*, keeps track of the sum of the signals received from the beginning of the process. The transition from a time step to the next obeys the following rules.

Every node *j* activated between $t - \Delta t$ and *t* sends at time *t* one signal to each of its neighbors through its outcoming links; no further signals will be sent by such an activated node at later times. The variable $V_i(t)$ of each target node at rest is incremented by the sum of the number of signals it has received. If $V_i(t)$ is greater than or equal to an activation threshold m_i , the node *i* switches from the state at rest (at time *t*) to the state active (at time $t + \Delta t$).

Once a node has been activated it remains in such a state until the end of the process, but fired only once at the time step of its activation. Hence, calling $s_i(t) = \Theta(V_i(t) - m_i)$ the state of a node *i* at time *t* ($s_i(t) = 1$ if the node is active, $s_i(t) = 0$ otherwise), the transition from a time step to the next is given by the following relation:

$$s_i(t + \Delta t) = s_i(t) + (1 - s_i(t)) \cdot \Theta\left(\sum_j a_{ij}s_j(t) - m_i\right)$$
(1)

where Θ designates the Heaviside function and the sum runs over the incoming links of the node *i*.

In the original version of this integrate and fire model, each node has the same activation threshold (quorum) *m*, each signal the same profile and each link has the same weight. Moreover this model describes complex systems where the time step Δt is assumed to be much smaller than every characteristic time associated with other features. It is worth noticing that the quorum percolation model is very flexible: more sophisticated versions of this model taking in account additional relevant biological features can be implemented. For instance, the fact that the membrane of a neuron cannot be modeled as a perfect capacitor since it is continuously leaking ions leads to a decay of its potential with some time constant τ ; it has been recently shown that the incorporation of such a decay in the model destroys criticality [8] (in the thermodynamical limit). From a statistical physics point of view, the process described by Eq. (1) converges towards a stationary state described by the fraction Φ of active nodes at equilibrium. On the other hand, in the framework of a mean field approach, such a stationary state can be viewed as one of the solutions which satisfy a self consistency equation [5,9]: the probability for a node to be active at equilibrium is the sum of the probability for a node to be initially activated and the probability to be connected to at least *m* active "in-neighbors" if not initially activated. Hence, the self consistency equation reads:

$$\Phi = f + (1-f) \sum_{k=m}^{\infty} p_k \sum_{l=m}^{k} {k \choose l} \Phi^l (1-\Phi)^{k-l}.$$
(2)

A truncated binomial appears because nodes that have less than *m* incoming links remain forever inactive (unless they were initially activated). Moreover, we will restrict ourselves to topological properties of the network described by a Gaussian in-degree distribution p_k with a mean value \bar{k} and standard deviation σ as observed in dissociated neuronal cultures [3]. When solving Eq. (2) in the physically meaningful range [0, 1] of the variable *f*, two regimes can be distinguished according to the value of the parameter *m*: For *m* smaller than a critical value m_c there is a range $f \in]f_0(m), f^*(m)[$ where three different real values of Φ satisfy Eq. (2), while for $m > m_c$, Φ is an increasing monotone function of *f*. Since the rules stated above require Φ to be an increasing function of *f* also when $m < m_c$, the behavior of Φ resolves the existence of an unstable branch in the range $[f_0(m), f^*(m)]$ in the following way: Φ follows the stable solution associated with the lowest branch for





Fig. 1. Phase diagram of the quorum percolation model in $\{f, m, \Phi\}$ coordinates. Below a critical value m_c of the quorum, the activity Φ of the network exhibits a sudden jump associated with a giant cluster for a value $f^*(m)$ of the initial activity; in the present case $\bar{k} = 50$ and $\sigma = 10$.

 $f \in [f_0(m), f^*(m)[$ in the phase diagram; the unstable solution and the lowest stable solution merge when f reaches $f^*(m)$, leading to a discontinuity in Φ . Such a sudden jump occurring at $f^*(m)$ in the global activity of the network is associated with a percolation transition on the graph \mathcal{G} where an infinitely small variation of the control parameter f results in the appearance of a so-called *m*-connected giant cluster, whose (normalized) size g(m) is given by the difference between the two remaining solutions of the self consistent equation at $f^*(m)$. Fig. 1 shows a typical phase diagram in $\{f, m, \Phi\}$ coordinates where the percolation transition can easily be seen. The resolution of an instability by a discontinuity below a critical point is well known to occur in the case of the liquid vapor transition described by the Van der Waals equation of state. It should be pointed out that the unstable solution branch is resolved in a quite different way from the quorum percolation model since the position of the discontinuity is determined by the Maxwell construction; moreover, from a mean field point of view, the polynomial self consistent equation has a much more simple structure than in the present case. Following the usual concepts of percolation on physical lattices [1] g(m) can be considered as an order parameter whose behavior in the vicinity of m_c is driven by a power law $g(m) \sim \left(\frac{m_c-m}{m_c}\right)^{\beta}$. On top of the resolution of Eq. (2), the study of such a critical behavior, namely the

driven by a power law $g(m) \sim \left(\frac{m_c - m}{m_c}\right)$. On top of the resolution of Eq. (2), the study of such a critical behavior, namely the calculation of the exponent β , requires (2) a precise value of m_c . Since we deal with a discrete model, m_c cannot be given with a better precision than one unit; one could imagine to overcome this problem by setting high values of k in order to increase the relative precision on m_c . This option would need to handle huge binomial coefficients, so that it is not possible to implement it numerically; moreover very high values of \bar{k} are not observed in dissociated neuronal cultures. Keeping in mind the idea that, in a more sophisticated version of the model, m should not necessarily remain integer if disorder is taken in account by varying the threshold from a node i to another one or by varying the signal value according to some probability distributions, we work out a prolongation of the quorum percolation model to continuous values of m. This can be done by introducing the beta functions $B(x, y) = \int_0^1 t^{x-1} (1-t)^{y-1} dt$ and the incomplete beta functions $B_{\phi}(x, y) = \int_0^0 t^{x-1} (1-t)^{y-1} dt$; it can be shown [10,11] that a truncated binomial can be written as the ratio of an incomplete beta function to a beta function:

$$\sum_{l=m}^{k} \binom{k}{l} \Phi^{l} (1-\Phi)^{k-l} = \frac{B_{\phi}(m, k-m+1)}{B(m, k-m+1)}.$$
(3)

The prolongation of the self consistent equation to non integer values of *m* is then straightforward; it reads:

$$\Delta = f + (1 - f) \int_{m}^{\infty} \frac{1}{\sqrt{2\pi\sigma}} e^{\frac{-(k-\bar{k})^2}{2\sigma^2}} \frac{B_{\phi}(m, k - m + 1)}{B(m, k - m + 1)} \cdot dk - \Phi = 0.$$
(4)

The resolution of such an equation is tricky from a numerical point of view; we first solved it with the help of the software Mathematica which allows to work with arbitrary precision. Since Mathematica is able to deal with huge binomial coefficients, we ensured in several cases that the numerical results obtained in the framework of continuous and discrete versions were perfectly consistent. Unfortunately the computing time is prohibitive and prevents an extensive study with the help of Mathematica. The computational method we implemented finally calls routines provided by the numerical library NAG [12]: G01EEF enables to evaluate the beta functions B(m, k-m+1) and $B_{\phi}(m, k-m+1)$, and D01GAF calculates

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Fig. 2. Evolution of Δ as a function of Φ for different values of the initial activity $f \in [0.1, 0.9]$ and fixed values m = 30, $\bar{k} = 50$ and $\sigma = 12$. Below $f^*(m) \simeq 0.368$ the self consistency equation has three solutions Φ_s (when $\Delta = 0$); the lowest solution and the unstable one move closer as f increases, until they merge at $f^*(m)$.

the integrals using a robust third order finite-difference method due to Gill and Miller including an error estimate [10]. Additional comparisons between the two numerical methods led us to ensure a coherent accuracy by setting a cutoff on the upper bound of the integral at $m + 5\sigma$. The equation $\Delta = 0$ is solved at fixed values of k, σ , m, and f, leading to one or three solutions Φ_s with a relative precision at least smaller than 10^{-4} ; the procedure is repeated iteratively, the value of f being driven by an adaptive dichotomy process until the value of $f^*(m)$ is reached within a desired precision; g(m) can be easily deduced from the difference between the degenerate solution (when the two smallest roots merge) and the upper stable one. The behavior of Δ as a function of Φ when f increases is shown on Fig. 2 where the two regimes above and below $f^*(m)$ together with the value of g(m) can easily be seen. The estimation of m_c is done by exploring a range of m also driven by a dichotomy process; such a procedure is tricky and must be carefully carried out: m_c is the limiting value of m where the width of the domain $[f_0(m), f^*(m)]$ associated with three solutions of the equation $\Delta = 0$ tends towards zero. Now, the width of this domain decreases when approaching the critical value m_c from below; in order to avoid missing values of *m* associated with three solutions, one has to decrease the step in *f* simultaneously with the dichotomy in *m*. It is worth noticing that the direct resolution of the self consistent equation, besides its ability to deal with non integer values of m, enables to get rid of a finite size scaling analysis as usually necessary by means of Monte-Carlo methods. In the case of second order percolation transitions, such an analysis allows to calculate critical exponents from the behavior of the moments of the cluster size distribution as a function of the system size [1]. It is based upon the hypothesis that the behavior of the moments in the critical region depends upon the ratio of the system size L to the correlation length, ξ , through scaling functions. Such an analysis is not straightforward in the case of a first order percolation transition. The self consistency equation provides a direct access to the thermodynamical limit.

2. Results

The connectivity parameters of the graphs we deal with lie in the range associated with cultures of dissociated neural networks; we investigated thoroughly the mean numbers of incoming links $\bar{k} = 50$ and $\bar{k} = 100$ for different values of the width σ of the Gaussian distribution. Typical curves showing the evolution of the solutions of Eq. (4) right at the value $f^*(m)$ where the percolation occurs are shown on Fig. 3. High precision values of m_c are necessary to calculate the critical exponent β in a reliable way; these values, obtained from intensive numerical computations are reported in Table 1. The limiting case where the width of the distribution p_k tends towards zero corresponds to regular graphs with exactly \bar{k} incoming and outcoming links per node; hence, if $m > \bar{k}$, the information cannot spread throughout the network and we have $\Phi = f$, while $\Phi = 1$ if $m \le \bar{k}$. Thus m_c tends towards \bar{k} as $\sigma \to 0$. The behavior of the critical thresholds is more conveniently described with the help of the reduced quantity m_c/\bar{k} : curves showing the evolution of m_c/\bar{k} as a function of the relative width σ/\bar{k} of the distribution are plotted on Fig. 4. As long as σ/\bar{k} is small enough, the curves collapse together, so that m_c/\bar{k} depends only on σ/\bar{k} , independently of \bar{k} . It is therefore convenient to express the difference between m_c/\bar{k} and 1 as a series of the relative width of the incoming links distribution. The fits we performed lead to :

$$\frac{m_c}{\bar{k}} = 1 - A\left(\frac{\sigma}{\bar{k}}\right) + B\left(\frac{\sigma}{\bar{k}}\right)^2 \tag{5}$$

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Table 1

High precision values of <i>m_c</i> associated	with different incom-
ing links Gaussian distributions.	

	k	σ		m _c		
	50	3	3	46.312	9(2)	
	50	4	1	45.264	6(2)	
	50	5	5	44.283	3(2)	
	50	6	5	43.363	1(2)	
	50	7	7	42.506	42.5067(2)	
	50	8	3	41.712	0(5)	
	50	9	Ð	40.973	3(2)	
	50	10)	40.295	1(2)	
	50	12	2	39.093	7(2)	
	50	13	3	38.569	05(5)	
	50	15	5	37.652	5(2)	
	100	1	3	96.177	4(2)	
	100	(j .	92.801	(1)	
	100	10)	88.773	0(2)	
	100	14	2	86.931	(1)	
	100	It)	83.652	(1)	
	100	20)	80.890	(1)	
	100	24	1	78.446	(1)	
	100	20)	70.308	0(2)	
$1 \frac{\Phi_s}{1}$					*****	
		• • • • • •		σ=3		
0.8					***	
0.0		σ=	8	A A A A A		
0.6			*			
0.6	σ=15					
0.4	34 3	6 38	40	<u></u> 12 14	46	m 48
52	51 5	0 50	10		10	10

Fig. 3. Solutions $\Phi_{\underline{s}}$ of the self consistency equation right at $f^*(m)$ as a function of the quorum *m* for three different values of the width σ of the Gaussian distribution p_k and k = 50. The lower branches are associated with the merging of the lowest solution and the unstable one at $f^*(m)$. The critical value m_c is located at the common value of the upper and lower branches when the slope becomes infinite.

with A = -1.2933(68), B = 1.585(29) for $\bar{k} = 50$ and A = -1.2748(63), B = 1.565(28) for $\bar{k} = 100$. The behavior of m_c can be understood according to the following heuristic argument, illustrated on Fig. 5: for a fixed value of σ/\bar{k} , m_c moves away from \bar{k} as \bar{k} increases in such a way that the surfaces colored in gray on Fig. 5 below the Gaussian distribution of

incoming links remain practically constant. Therefore, the surface given by $f_{\min} = \int_{m_c}^{\infty} \frac{1}{\sqrt{2\pi\sigma}} + e^{\frac{-(k-\bar{k})^2}{2\sigma^2}} dk$ represents a lower bound of the fraction of nodes of the network liable to be involved in the activity of a percolation process, since their number of incoming links is greater than any quorum allowing the growth of a giant cluster; as far as σ/\bar{k} is fixed f_{\min} is practically constant. It should be pointed out that f_{\min} is rather high, since we find $f_{\min} \simeq 88\%$ when $\sigma/\bar{k} = 0.1$ and $f_{\min} \simeq 76\%$ when $\sigma/\bar{k} = 0.24$.

We are now able to calculate numerically the critical exponent β for different values of \bar{k} and σ over the accessible numerical range. Figs. 6 and 7 show the variation of the size of the giant cluster g as a function of $\left(\frac{m_c-m}{m_c}\right)$. In any case, these variations fit power laws with a very good reliability over a range of the relative distance to the critical points between 10^{-4} and 10^{-1} . The error bars grow rapidly when approaching closer to m_c and hinder the reliability of the fit. It should be pointed



Fig. 4. Evolution of the critical values m_c as a function of the width of the incoming links distribution normalized by \bar{k} .



Fig. 5. Two distributions of incoming links sharing the same value of σ/\bar{k} , $\bar{k} = 50$, $\sigma = 5$ and $\bar{k} = 100$, $\sigma = 10$. The gray colored surfaces represent the fraction of nodes with a number of incoming links smaller than m_c , thus unable to be involved in the activity of the network at the critical point, unless they have been set active at t = 0.

out that the fit of g as a function of $\left(\frac{m_c-m}{m_c}\right)$ is very sensitive to the value of m_c and moves away from power laws when exploring a range of width about ten times the errors bars around the values we calculated. Hence the precision needed on m_c is in agreement with the numerical calculations we carried out. The values of the associated critical exponents β are reported in Table 2.

A dynamical system analysis of the self consistent equation (4) $G(\phi, m, f) = f + (1-f)F(\phi, m) - \phi = 0$ enables to obtain the normal form associated to the transition, here assimilated to a pitchfork bifurcation, by expanding *F* in series right at the critical point (ϕ_c , m_c). Such an expansion leads to a cubic equation involving coefficients which exhibit a very complicated form because $F(\phi, m)$ is a high degree polynomial involving some huge binomial coefficients. Such an equation leads to the classical result $\beta = 1/2$. Mainly, we deduce from our results that the power law 1/2 is only valid in the extremely close vicinity of the critical point, probably due to the complex structure of $F(\phi, m)$. In the accessible range approaching the 57



Fig. 6. Evolution of the size of the giant cluster g(m) as a function of the relative distance to the critical points for $\bar{k} = 50$ and four different values of σ .



Fig. 7. Evolution of the size of the giant cluster g(m) as a function of the relative distance to the critical points for $\bar{k} = 100$ and three different values of σ .

Table 2

Critical export coefficient of	the fit. β for different the fit.	values of \bar{k} and σ ;	R is the reliability
k	σ	β	$1 - R^2$
50	3	0.464(7)	$3.5 imes 10^{-4}$
50	8	0.466(6)	$1.4 imes 10^{-4}$
50	13	0.479(3)	$0.5 imes 10^{-4}$
50	15	0.489(5)	$0.5 imes 10^{-4}$
100	3	0.473(8)	$22 imes 10^{-4}$
100	10	0.464(6)	$10 imes 10^{-4}$
100	28	0.487(6)	1×10^{-4}

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critical point until $\left(\frac{m_c-m}{m_c}\right) \sim 10^{-3}$ it turns out that the effective critical exponent β varies slightly with \bar{k} and σ . Such an effective non universal behavior reminds of the weak universality studied by one of us in the context of fractal networks [13]. Hence, from a theoretical point of view, the study of the critical behavior, namely the values of m_c and β should enable to infer the connectivity of the network that is the values of \bar{k} and σ .

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Memory decay and loss of criticality in quorum percolation

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In this paper, we present the effects of memory decay on a bootstrap percolation model applied to random directed graphs (quorum percolation). The addition of decay was motivated by its natural occurrence in physical systems previously described by percolation theory, such as cultured neuronal networks, where decay originates from ionic leakage through the membrane of neurons and/or synaptic depression. Surprisingly, this feature alone appears to change the critical behavior of the percolation transition, where discontinuities are replaced by steep but finite slopes. Using different numerical approaches, we show evidence for this qualitative change even for very small decay values. In experiments where the steepest slopes can not be resolved and still appear as discontinuities, decay produces nonetheless a quantitative difference on the location of the *apparent* critical point. We discuss how this shift impacts network connectivity previously estimated without considering decay. In addition to this particular example, we believe that other percolation models are worth reinvestigating, taking into account similar sorts of memory decay.

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I. INTRODUCTION

The model of quorum percolation [1] (QP), which corresponds to bootstrap percolation where links are directed rather than symmetrical [2], has been recently proposed to understand the collective behaviors of neuronal cultures, by reproducing the step by step propagation of activity throughout networks of neurons [see Fig. 1(a)]. Although such percolation models are also relevant to other gated signal propagation processes such as the spread of disease, rumors, and opinions, we will focus our discussion on cultured neuronal networks, the original scope of QP.

In this framework, an inactive neuron becomes active if at least *m* of its "in-neighbors" (those found following back up the incoming directed links) are active themselves, or from another perspective if it has accumulated at least m signals sent by "in-neighbors" when they became active. Once they reach the threshold, neurons remain active until the end of the percolation process; however, only one signal is sent through each of their outgoing links. Depending on the value of the quorum *m*, the final active fraction ϕ at the end of the percolation process is either a continuous or discontinuous function of the fraction f initially set as active. Let us remark that we look here at responses of the entire network upon simultaneous stimulation of many neurons; we are consequently in a regime quite different from the one in studies of avalanches propagating from single neurons in the context of self-organized criticality [3]. The $\phi(f,m)$ graph [see Fig. 1(b)] and especially the critical value m_c , defined as the last *m* for which ϕ_m is discontinuous (ϕ_m is a continuous function of f if $m > m_c$), can be correlated to the features of the network connectivity, and comparison with data obtained experimentally from neuronal cultures was used to infer biologically relevant information such as the evolution in the number of synapses per neuron during development, as well as the fraction of inhibitory neurons in the culture [4].

However, this model lacks important properties of biological neurons which can affect the expected behavior of networks; one of them is decay, or leakage of accumulated signals. Indeed, the membrane of biological neurons can be compared to a capacitor that supports electric potential difference through ionic charge separation. Active neighbors will inject ionic currents into this capacitor, changing the electric potential difference across the membrane until it eventually passes a threshold value (associated with m in the framework of the QP model): the neuron fires. But in reality, the membrane is not a perfect capacitor as it is continuously leaking ions; the membrane potential eventually decays exponentially to its resting value with a time constant τ . If the time interval between each received signal is significantly larger than τ , they will not add up and make the neuron fire. The state of a real neuron is thus not only determined by the number of received signals, but also by the timing of arrival of these signals. Particularly, this effect can not be neglected during the initiation of bursts, where the leader (or first-to-fire) neurons receive sparse signals from the rest of the network [5]. Although decay is possibly relevant also beyond the context of dissociated neuron cultures to other systems it has, to our knowledge, never been studied in this model or in the more general topic of percolation theory. Here, we investigate an extended model of QP that includes decay, in which each discrete accumulated signal can disintegrate independently from the others with a probability $d \in [0,1]$ at each step of the percolation process.

The evolution of the neuronal activity in this network is described by a discrete time stochastic process with a time step Δt involving two competing mechanisms: on the one hand the reception of new signals sent by activated neurons, on the other hand the decay of the accumulated signals with a characteristic time τ .

The sum of accumulated signals (or potential) decays on average exponentially with a time constant $\tau = -\Delta t/\ln(1 - d)$, as the membrane potential would do in biological neurons. If we normalize the time constant τ to the duration of an iteration *in vitro*, that is, the minimal time interval necessary to transmit activity from a neuron to another, we can reckon the *in vitro*

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FIG. 1. (Color online) Illustration of quorum percolation and decay. (a) Successive frames taken during QP showing how the inactive neurons (in light gray) become active (in orange) as soon as their potentials reach m. In this particular example, m = 3. (b) Relationship between f, ϕ , and m in the classical QP model, for a Gaussian "in-connectivity" with $\bar{k} = 50$ and $\sigma = 10$. f is the initial active fraction (externally activated) and ϕ is the fraction of active neurons at the end of the cascade. m defines the number of active "in-neighbors" necessary for a neuron to become active if it was not part of f. For $m \leq m_c$, we observe a first order transition in ϕ . The height of the jump at the discontinuity is called g(m) (see text). (c) Schematic representation of QP with decay. Random disintegration of signals (decay) between steps (glowing links) changes the final state of the network although the initial configuration is the same as in (a).

value of the decay parameter *d*. Taking into account the action potential duration and propagation speed, the size of a typical culture and the synaptic delay, we can estimate the duration of an *in vitro* time step at 1 ms. Knowing the range of value for τ , estimated at 7 to 120 ms depending on the type of neuron [6,7], we find that *d* lies approximately between 0.1 and 0.01.

Previous works on QP included two approaches. On the one hand, one used explicit Monte Carlo simulations in which each neuron is individually simulated in a stochastic process according to the QP rules stated above; from a statistical physics point of view, such a process converges towards a stationary state. On the other hand, this stationary state can be described as the solution ϕ of a self-consistency equation [1,4] representing the relation between f, ϕ, m and the "in-connectivity" distribution (p_k is the probability that a random node has k inputs). Indeed, the probability for a neuron to be active at equilibrium (i.e., belonging to ϕ) corresponds to the probability for a neuron to be either active through initial stimulation or, if not externally activated, to be connected to at least *m* active "in-neighbors." Neurons that have less than m inputs remain inactive (unless they were initially activated) and are not taken into account in the equation. If p_k denotes the probability that a random neuron has k inputs, the self-consistency equation reads as

$$\phi = f + (1 - f) \sum_{k=m}^{\infty} p_k \sum_{i=m}^{k} \binom{k}{i} \phi^i (1 - \phi)^{k-i}.$$
 (1)

For $m \leq m_c$, the sudden disappearance of one of the stable solutions of Eq. (1), occurring when f reaches f^* , results in a discontinuity in ϕ . The existence of a discontinuity in the absence of decay is consequently guaranteed independently from numerical approximations.

Although it is possible to implement decay in explicit Monte Carlo simulations, we can not write self-consistency equations to determine fixed points (ϕ) in the presence of decay. Nevertheless, from a mean field point of view, we were able to establish a recursive relation enabling us to fully describe the stochastic dynamics in the presence of decay. The striking point is that decay appears to change the properties of the network all along the process. Thus, the whole trajectory as a function of time can be solved. In this paper, we make use of the two numerical approaches mentioned above to show how the decay changes the critical behavior of the network. We conjecture from numerical evidence which is based on the resolution of steep but finite slopes that even infinitely small decay kills the first order transition (ϕ is always a continuous function of f) for m > 1, that is, $m_c = 1$ independently of the connectivity.

II. MATERIAL AND METHODS

A. Explicit Monte Carlo simulations of quorum percolation

This implementation involves a finite size network of N neurons, whose states are individually computed according to the state of each neuron at the previous step.

Network construction. The connectivity is entirely determined by the "in-distribution," the distribution of the number of incoming links per neuron. For each neuron in the network, this number is randomly chosen according to this distribution (probability to choose k is p_k), and the origins of these incoming links are then chosen randomly amongst the N-1other neurons. Because the origin of each link is chosen randomly, the "out-distribution" is independent from the "indistribution" and follows a binomial law $\mathcal{B}[(N-1)k, \frac{1}{N}]$ <u>[]</u> under the condition that the mean of the in-distribution \bar{k} is defined. Note that the resulting network is not endowed with any metric, which deems it acceptable to model small and dense networks where the range of connection is significantly greater than the dimensions of the whole network. The validity of this approximation for real cultures is discussed in detail in other studies [8].

Iterative propagation of activity during cascade response. At step 0, the potential (i.e., the sum of accumulated signals) is set to *m* for a fraction *f* drawn randomly in the network. To make one iteration, the algorithm loops over all the neurons in the network, and the potential of inactive neurons is compared to the quorum *m*. If it is greater or equal to *m*, the neuron becomes active and the potential of its out-neighbors is incremented by one through a buffer that is necessary to synchronize iterations. If the potential is submitted to a Bernoulli trial of parameter *d* (with $0 \le d \le 1$) and is disintegrated if the trial is positive; the potential has decayed. The buffer of each



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FIG. 2. (Color online) Representation of a mean field network. (a) Shows the different $p_{keq,s}$ that constitute the network at a given time step. Note that the actual sizes of the active and inactive fractions are independent from the number of squares that constitute them. (b) Pictorial description of Eq. (4) showing how it is applied to two different $p_{keq,s}$ displayed as green filled squares. Their values at step t + 1 are computed from their previous state and those of a subset of $p_{keq,s}(t)$, displayed as gray hatched squares. Neurons "inside" those squares which lose through decay and receive from incoming neighbors the right amount of signals will become equivalent to the neurons in the associated green square at the end of the iteration. For one of the hatched squares, outlined in yellow, these two antagonist effects are represented by a first red arrow for the decay and the associated decrease of k_{eq} [see Fig. 2(c)], and a second outlined blue arrow for the reception of new signals. (c) Equivalence between neurons. The two neurons in the middle have the same probability to become active in the next step.

inactive neuron is subsequently transferred to the potential, and a new iteration begins. The process can go on as long as the number of active neurons keeps increasing. The final value of the active fraction is ϕ .

B. Mean field simulations of quorum percolation

As already mentioned above, when we introduce decay in the model, it is no longer possible to calculate ϕ with the self-consistency equation previously described [Eq. (1)]. It is, however, possible to perform a mean field iterative computation of activation cascades, as one would follow the progress of percolation in an explicit numerical simulation. In this model, there are no individual neurons; the trick consists in partitioning the network in suitable subsets that group together neurons of a same kind, i.e., neurons that would be indistinguishable in an explicit network regarding their probability of activation. The algorithm computes the relative size of the different fractions step by step, from which we can extract the time evolution of the active fraction's size and its limit ϕ .

Representation of a mean field network. At each time step, the nodes of the network are partitioned in subsets of the same kind defined by a triplet (k,x,s) where k is the number of inputs, x is the number of active in-neighbors or the number of used incoming links, and s is the potential. Note that without decay, it is not necessary to distinguish the potential of a given inactive neuron from the number of its active in-neighbors because x and s are equal.

The future state of the neurons in each of these partitions is completely determined by how many signals they already have s, and how many signals they are going to receive next, which depends on the number of unused incoming links k - x (the x used incoming links, through which signals have already been received, can not bring new signals to the target neuron since the upstream neurons had already fired). Consequently, neurons defined by two different triplets (k_1,x_1,s_1) and (k_2,x_2,s_2) would have the same fate as long as $s_1 = s_2$ and $k_1 - x_1 = k_2 - x_2$.

The two triplets (k,x,s) and (k - [x - s],s,s) satisfy these conditions and thus are strictly equivalent although the second one has virtually experienced no decay at all at this particular time step [see Fig. 2(c)]. We can hence describe each fraction by a couple (k_{eq},s) instead of a triplet, where $k_{eq} = k - (x - s)$, and the whole network can be conveniently represented by a two-dimensional (2D) table containing the size of the different possible equivalent fractions $p_{k_{eq},s}$ [see Fig. 2(a)]. In this table, the sum of $p_{k_{eq},s}$ with s < m gives the size of the inactive fraction of the network at step t, U_t .

Iterative computation of the network response. To understand how we derived the iterative relation, let us consider a two-step mechanism, in which the first stage describes decay (from time t to t_d) and the second stage the reception of new signals (from step t_d to t + 1). For the decay [red arrow on Fig. 2(b)], the equivalence principle stated above implies that neurons defined by the couple ($k_{eq} + j, s + j$) at time t will be in the new configuration (k_{eq}, s) after decay of j signals. The relevant partitions to compute $p_{k_{eq},s}(t_d)$ are thus all the different $p_{k_{eq}+j,s+j}(t)$ with $j \ge 0$ (decay can only decrease the number of accumulated signals) and $j \le m - 1 - s$ (only inactive neurons undergo decay):

$$p_{k_{eq},s}(t_d) = \sum_{j=0}^{m-1-s} p_{k_{eq}+j,s+j}(t) \binom{s+j}{j} d^j (1-d)^s.$$
(2)

Note that in the limit d = 0, $p_{k_{eq},s}(t_d) = p_{k_{eq},s}(t)$, i.e., this step does not do anything. The second part of the iteration describes

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the reception of new signals [blue arrow on Fig. 2(b)]. The neurons characterized by the couples $(k_{eq}, s - i)$ at time t_d will build up the partition $p_{k_{eq},s}(t + 1)$ by receiving *i* new signals through their remaining $k_{eq} - (s - i)$ unused incoming links:

$$p_{k_{eq},s}(t+1) = \sum_{i=0}^{s} p_{k_{eq},s-i}(t_d) \binom{k_{eq} - (s-i)}{i} \\ \times \left(1 - \frac{U_t}{U_{t-1}}\right)^i \left(\frac{U_t}{U_{t-1}}\right)^{k_{eq}-s}$$
(3)

with $(1 - \frac{U_t}{U_{t-1}})$ being the probability that the neuron following back up any unused incoming link was activated exactly at step *t* and consequently sent a signal which is received during this iteration (this probability can be seen as the conditional probability that a neuron does not belong to U_t given it belongs to U_{t-1}). Equations (2) and (3) combine in the following recursive relation:

$$p_{k_{eq},s}(t+1) = \sum_{i=0}^{s} \left(\sum_{j=0}^{m-1-(s-i)} p_{k_{eq}+j,s-i+j}(t) {s-i+j \choose j} d^{j}(1-d)^{s-i} \right) \times {k_{eq}-(s-i) \choose i} \left(1 - \frac{U_{t}}{U_{t-1}} \right)^{i} \left(\frac{U_{t}}{U_{t-1}} \right)^{k_{eq}-s}.$$
 (4)

At step t = 0, all neurons are inactive $(U_0 = 1)$ with a potential of 0. We set then the $p_{k_{eq},0}(0)$ of the table according to the p_k distribution. After the external activation of a fraction f between steps 0 and 1, we have $U_1 = 1 - f$ and because neurons in f are recruited independently of their kinds, we have simply $p_{k_{eq},0}(1) = (1 - f) \times p_{k_{eq},0}(0)$. From this point, we can compute all the $p_{k_{eq},s}(2)$ and U_2 using Eq. (4), and so on. It is important to mention that in the absence of decay we can derive from this relation the same self-consistency equation (1) originally proposed in the model without decay (see Supplemental Material [9], Sec. 1). This confirms that our model is consistent with previous work and that we do expect a discontinuity when d = 0 with this algorithm. In particular cases, we can also derive a simple analytical expression for the time evolution of the system (see Supplemental Material [9], Sec. 2).

The limits of $p_{k_{eq,s}}$ reached theoretically after an infinite number of iterations provide the size of the final activated fraction. In practice, we can continue to iterate until the active fraction grows slower than a stopping criterion ν (with $0 < \nu < 1$) and define the final fraction activated as $\phi = 1 - U_{\text{last}}$. Although using the difference between two consecutive iterates is generally not a good criterion for convergence (it is rather indicating stagnation), it is in this case the best one to compare with explicit simulations (where the stopping criterion is equivalent to 1/N). In addition, we can show in the case d = 0 that despite the fact that calculations of single ϕ are infinitely sensitive to ν close to the discontinuity, the error made on the size of the discontinuity itself is simply proportional to ν when ν tends towards 0 (see Supplemental Material [9], Sec. 3), which makes it a good stopping criterion regarding the sizes of the jumps.

The two algorithms, explicit and mean field, are used to generate data of the response ϕ of a particular network, as a

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function of the size of the fraction f initially activated, the quorum m, and the decay parameter d.

C. Network response analysis

We detail here the numerical method key for the determination of the presence and size g(m) of discontinuities in $\phi(f,m)$, which is necessary to characterize the percolation transition and reveal properties on networks connectivities. Let us consider a particular quorum *m* and look at the function ϕ_m which associates *f*, the initial fraction of neurons activated, to $\phi_m(f)$, the final fraction activated. Let us call Δ_m the function which associates for each *f* the difference between the right limit of ϕ_m in this point and its actual image $\phi_m(f)$ (the function is defined everywhere)

$$\Delta_m(f) = \lim_{\epsilon \to 0^+} \phi_m(f+\epsilon) - \phi_m(f).$$
(5)

By definition, Δ_m is null where ϕ_m is continuous and nonnull at each point of discontinuity. Since ϕ_m is an increasing function, Δ_m is positive and we can define

$$g(m) = \max[\Delta_m(f)] \tag{6}$$

as the size of the largest discontinuity (zero meaning there is no discontinuity). Note that in the case of a Gaussian connectivity at least, and in the limit of a null decay, the self-consistency equation reveals that there is at maximum one discontinuity for each m, which is consequently returned by the g function without ambiguity. As supported by extensive simulations, we consider this to be still valid in the presence of a non-null decay.

This function g is crucial to infer information about the network, but it can not be computed analytically. In practice, we can only approximate this function by another function g_{ϵ} defined by

$$g_{\epsilon}(m) = \max_{f} [\Delta_{m,\epsilon}(f)], \tag{7}$$

where
$$\Delta_{m,\epsilon}(f) = \phi_m(f+\epsilon) - \phi_m(f)$$
 (8)

that we call the *apparent* size of the discontinuity for a given value of ϵ .

This unavoidable approximation leads to an issue for the determination of m_c . Indeed, this theoretical value is defined as the maximal *m* value for which g(m) > 0, but in numerical approximations, we know that $g_{\epsilon}(m) \ge \epsilon > 0$ for any *m* value. The quality of the approximation of m_c is consequently dependent on an arbitrary choice for the accuracy ϵ . Although we do not address this issue in this paper, one could simply define a threshold below which the size of the discontinuity is considered as null. Finally, an important remark is that in a network of finite size *N*, the smallest possible value for ϵ is equal to the stopping criterion $\nu = 1/N$. In the mean field algorithm, we can arbitrarily choose the values for ϵ and ν , but we necessarily chose the stopping criterion ν to be equal to, or lesser than, ϵ to avoid irrelevant approximations.

III. RESULTS

A. The mean field algorithm is equivalent to an infinite network

We tested the two numerical implementations of the quorum percolation model for different decays and connectivities. For

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FIG. 3. (Color online) Comparison of explicit and mean field simulations. Lines are computed using the mean field algorithm while points of the same color correspond to 10 different equivalent explicit simulations. As N increases from (a) 1000 to (b) 100 000, explicit simulations converge to the mean field behavior. $\bar{k} = 50$, $\sigma = 10$, d = 0.1.

a wide range of decay values and Gaussian p_k distributions $(\bar{k} = 25, 50, 75, 100 \text{ and } 0 \le \sigma \le \bar{k}/3)$, explicit simulations tend towards the mean field simulations as *N* increases and the possible finite size effects (loops, high variability within small sets of neurons, reduced *f* resolution) are getting negligible. The convergence of the behavior of large explicit networks to the mean field description is illustrated in Fig. 3. Since the mean field network representation captures the behaviors of explicit networks and that we can not technically work with explicit networks bigger than 10^6 neurons (imposing limitations on ϵ and ν), the mean field algorithm will be used to study the effects of decay in the rest of the simulations.

B. The decay reduces the apparent size of discontinuity

The size of the discontinuity g, as a function of m, was previously used [1,5] to infer connectivity in real neuronal networks; since decay is part of the physics of these networks, we wanted to evaluate to what extent the decay parameter d was changing those results. To this aim, we calculated the apparent size of the discontinuity g_{ϵ} for different d at constant Gaussian connectivity. For an arbitrary value of ϵ , $g_{\epsilon}(m)$ decreases when d increases, with a resulting apparent downwards shift in m_c , as shown in Fig. 4. This figure also indicates how the apparent size of the discontinuity and consequently m_c are dependent on ϵ . This latter effect is discussed in the next section.



FIG. 4. (Color online) Apparent approximate size of the discontinuity g_{ϵ} as a function of d for (a) $\epsilon = 10^{-3}$ and (b) $\epsilon = 10^{-10}$. The connectivity p_k follows a Gaussian distribution of parameters $\bar{k} = 25$ and $\sigma = 5$. $\nu = 10^{-10}$.

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FIG. 5. Apparent size of the discontinuity $g_{\epsilon}(2)$ as a function of ϵ , in a network where $\bar{k} = 25$, $\sigma = 5$, and d = 1 (immediate decay). What is interpreted as a very large discontinuous transition at larger ϵ vanishes completely when $\epsilon \approx 10^{-40}$. For each point, $\nu = \epsilon/10$. High accuracy computations were done with MATLAB and the VPA package.

C. Effect of decay on the real size of the discontinuity

In our attempt to give a good estimation of the real size of the discontinuity g necessary to characterize the percolation transition, we reduced ϵ expecting to find g_{ϵ} converging quickly to its limit g, as it is the case without decay. Surprisingly, when d is non-null, continuously decreasing ϵ leads to a steady gradual reduction of g_{ϵ} (see Fig. 5).

The convergence of $g_{\epsilon}(m)$ to 0 as ϵ tends towards 0 is faster when the decay *d* is strong and the m/\bar{k} ratio is high, but the phenomenon was observed in every computationally accessible case, as long as *d* was non-null. This strongly suggests that $g_{\epsilon}(m)$ always converges towards 0 when d > 0and m > 1. Note that if m = 1, the decay has no effect as neurons activate immediately and do not have a chance to lose any signal. In other terms, we hypothesize that when there is non-null decay, we have $m_c = 1$ independently from the network's connectivity.

IV. DISCUSSION

In the simple model of quorum percolation, originally introduced to describe the collective response of neuronal cultures to external stimulations, each neuron "integrates" the state of its neighbors over its incoming links, "fires" once a threshold m, or quorum, is reached, and eventually propagates a signal to other neurons. Taking the threshold m as control parameter, a transition characterized by the appearance of a discontinuity in the network response is visible as m becomes lower than a critical m_c . This transition is due to the coalescence of small m-connected groups of neurons into a single giant m-connected cluster spanning across the whole network.

Experimentally, *in vitro* 2D neuronal cultures display similar transitions when the population is treated with drugs that artificially increase the threshold m by reducing the synaptic strength in the entire network. Theoretical results of QP applied on networks with different in-degree distributions were used

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to infer the type of connectivity and the average number of synapses per neuron in those cultures. However, the correct inference of the connectivity depends critically on the validity of the model to describe what really happens in cultures.

With this in mind, we introduced in this paper an extension of the QP model referred to as decay QP (DQP) where signals integrated by neurons can be lost. This feature is motivated by a characteristic of natural neurons, that retain incoming signals information only over a duration set by the membrane time constant τ . In DQP, we model this decay as a stochastic process for each signal, with constant probability per time step to disappear *d*. In a network characterized by a membrane time constant τ and a time-step duration of Δt , we would have $d = 1 - \exp(-\Delta t/\tau)$.

Explicit Monte Carlo simulations of the DQP model are straightforward, but results are prone to finite size effects. In the case of classical QP, it was possible to characterize the true thermodynamic limit behavior at the equilibrium through a self-consistency equation which requires the p_{keq} distribution. Unfortunately, it can not be applied to the case of DQP because the p_{keq} distribution of the network [in which $x_{eq} = s$ and $k_{eq} = k - (x - s)$ for each neuron] changes in the course of the activation cascade.

To deduce the DQP behavior in the infinite limit, we introduced an alternative mean field simulation approach that iteratively computes the probabilities of neurons' states along a cascade of firings. The final fraction ϕ of the population responding to the initial stimulation of a fraction f can then be computed from the global network state reached after an infinite number of iterations, approximated numerically using a stopping criterion. The validity of this scheme is attested by the convergence of explicit Monte Carlo simulations to the mean field computations when increasing the finite network size.

For a given Gaussian random network defined by its mean connectivity and variance, we computed how the final fraction ϕ of responding neurons varies with the ignition fraction fand quorum m, for different decay values. The surfaces in the (ϕ, f, m) space obtained in this way appear to be qualitatively rather close to those obtained in the framework of classical QP [Fig. 1(b)], suggesting the same type of behavior and the existence of a critical point m_c also for DQP. However, closer inspection shows a very different picture since for DQP we find results depending on the discretization steps necessary to compute the response curves. When ϕ is computed on a denser grid, with smaller step size ϵ between successive values of f, the discontinuities which appear at a coarser scale are resolved in slopes with finite steepness. For each value of the decay we tested (down to $d = 10^{-4}$), we observed no convergence of $m_c(\epsilon)$ (as ϵ tends to 0) towards a value other than 1, where decay plays no role and hence where the first order transition persists. The observed apparent discontinuities in ϕ thus appeared to be dependent on the numerical accuracy used for the computation of the curves, gradually disappearing as $\epsilon \to 0$ when *m* was strictly greater than 1. Even in the case of m = 2, which is the situation with maximal resistance to decay, we observe the steady diminution of the size of the apparent discontinuity as ϵ decreases, leading to its eventual complete disappearance. This disappearance was observed in the case d = 1, at $\epsilon = 10^{-40}$ using arbitrary precision arithmetics (Fig. 5).

MEMORY DECAY AND LOSS OF CRITICALITY IN ...

We hypothesize that the disappearance of the discontinuous transition also happens for smaller decay values. We base this hypothesis on the idea that even an infinitely small decay becomes significant when the competing mechanism of receiving new signals (the rate of which is proportional to the growth rate) is also infinitely slow. Now, our argument is that in the classical case of discontinuous transitions, cascades initiated at $f^* + \epsilon$ will first present a temporary slowdown with a minimal growth rate equal to $\epsilon \times \frac{1-\phi(f^*)}{1-f^*}$ (see Supplemental Material [9], Sec. 2) before the explosive growth occurs. Since the reception of new signals is proportional to the growth rate, the effects of decay will increase as ϵ decreases, changing the course of the cascade to a greater extent. We imagine then how it is possible to tune the ratio between reception and decay (by choosing appropriate ϵ) so that the limit of the active fraction's size converges to any value normally forbidden by the discontinuous transition. However, the window around f^* . determined by the range of appropriate ϵ values, where this smooth transition occurs, becomes narrower as d decreases (ϵ values must be smaller and smaller). In the limit d = 0, this window would be reduced to a single point f^* , resulting in a discontinuous transition. This, with our numerical results. led us to conjecture that introduction of even infinitely small decay in quorum percolation destroys the first order transition for m > 1 and consequently that $m_c = 1$, independently from the connectivity.

This result raises the question as to whether our implementation of decay is correct pertaining the dynamics of in vitro neuronal networks. When we compare the membrane time constant to the synaptic delay, it appears that decay is not negligible in the case of a single signal transmitted from a firing neuron to another. But, in the original interpretation of QP, the active state in real neurons is assimilated to a train of 4 to 5 spikes during, in total, 20 ms. We can wonder if the multiple signals sent over the activity period are coherent with the idea of decay since it would regenerate to some extent the effect of an active neuron to its target. In reality, the signals, or excitatory post-synaptic potentials (EPSPs) in biological terms, which are transmitted successively to the post-synaptic neurons, diminish rapidly in a phenomenon called synaptic depression that is due to depletion of neurotransmitter vesicles [10]. We can speculate that such depression effect combines with the exponential decay in the post-synaptic neuron to produce a more complex but no less relevant decay: the incorporation of these effects in the model is an additional step towards a deeper understanding of collective behaviors in neuronal cultures.

Assuming the relevance of DQP as a valid paradigm for dissociated neuronal cultures, several factors can conciliate its noncritical behavior with experiments. Experimental curves for $\phi(f)$ include only a restricted number of data points, which is equivalent to the situation where ϵ is large (at best $\epsilon = 0.05$ experimentally for cultured neuronal networks [11]) and where we can interpret a steep slope as a discontinuity. In addition, increasing the experimental resolution would be eventually limited by the ultimate theoretical limit of 1/N in finite size networks. We conclude that DQP is compatible with neuronal culture experiments when finite size and finite accuracy in experiments and model are taken into account.

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We can wonder how much proper consideration of decay with DQP affects the prediction on cultured neuronal network connectivities that were inferred from experiments using only classical QP. As seen on Fig. 4 (and for a given resolution ϵ), the apparent critical value with decay $m_c(\epsilon, d)$ is lower than $m_c(\epsilon, 0)$ observed when there is no decay, and this effect increases as ϵ gets smaller. The consequence of this shift is that a model which does not take decay into account leads to an underestimation of m_c [by erroneously assimilating $m_c(\epsilon, 0)$ with $m_c(\epsilon, d)$]. Previous publications [1,5] make use of comparison between the experimental m_c^{expt} value and the theoretical QP m_c values obtained from simulations on various (\bar{k}, σ) Gaussian random networks to infer the biological network connectivity.

Simulations of QP in Gaussian networks suggest the relation $m_c \approx \bar{k} - \sigma$ [provided that σ is small compared to \bar{k} (unpublished result)]. This would mean that matching m_c^{expt} to the theoretical m_c obtained in a model without decay leads to an underestimation of \bar{k} at constant σ/\bar{k} . However, knowing the value of *d* in real networks would give the possibility to correct this estimation, which will be the purpose of future work.

V. CONCLUSION

The results of our mean field computations lead us to conjecture that the critical point expected from the classical QP model in the thermodynamical limit vanishes in the framework of the DQP model. They also reveal how the distinction between continuous and discontinuous transitions depends both on the size of the network and the accuracy in the control of external stimulation, and consequently that decay, although being part of the network's dynamics, may remain unnoticed. While many traditional applications of percolation models to statistical or steady state physics systems are interested in the behaviors of systems in the infinite size thermodynamic limit. it is worth noting that other uses may be better represented by a finite size case. It is remarkable that the study of the infinite limit of DQP leads to a qualitatively different interpretation of the behavior of neuronal networks subjected to decay, compared to what is obtained experimentally on accessible scales and resolutions in the domain of neuronal networks.

The conclusion in this paper about the absence of criticality in the thermodynamic limit of the DQP is still a conjecture founded on extensive numerical computations and two different implementations of the model. However, we believe that the mean field approach introduced here supplies a mathematical framework to study the effects of decay in a formal way, that can eventually bring indisputable proof of the disappearance of criticality in infinite networks subjected to decay.

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Discussion

Activation functions, representing the relationship between the input received by a unit and the amplitude of its response, is a critical concept in the field of artificial neural networks, as they define which computations are possible. Overly linear activation functions can not yield complex computation (linear combination basically), while saturation of non linear threshold or sigmoid units can prevent efficient learning (changing the input when a unit is saturated has no effect).

In the context where we assimilate neuronal populations to the units of an artificial neural network, the QP model allows to predict the shape of the activation function. A general result, captured in the self-consistent equation, is that the activation function of a population can be tuned by changing the excitability of individual constituents (captured by *m*) or the connectivity of the network (distribution of the number of synaptic neighbors per neuron).

In practice, *m* can easily be increased using chemical inhibitor, as it was done in experimental studies on QP [64], to reduce the strength of individual synapses. Excitability can also be modified by changing the medium formulation in general, in particular the concentrations of ions defining the value of the resting membrane potential (sodium and potassium [65]) and divalent cations like magnesium which can affect ion channels conductance [66]. Controlling several populations chemically would however become rapidly unrealistic for large networks, as it would require continuous and independent perfusion of different media on each unit. Moreover, the effects of such chemicals might be difficult to control over long periods of time because of different homeostatic mechanisms active inside neurons. For instance, neurons incubated with blockers of a particular receptor generally compensate by over expressing this receptor in order to maintain its function.

Affecting the connectivity of the population seems therefore to be a more pragmatic alternative to control the activation function of neuronal populations in the long term. Here I discuss the effects of connectivity and other experimental parameters, extending the initial model of QP, on the activation of neuronal populations.

Effects of connectivity

Experimentally, the most straightforward way of changing the connectivity is to control the plating density. If we hypothesize that on an infinite 2D culture, connections are only established between neurons separated by a distance smaller than *r*, then the number of neighbors in a culture of density *d* would follow a Poisson law of parameter $\bar{k} = d\pi r^2$. However, experiments showed that in cortical cultures neurons establish in average the same number of synapses over a wide range of plating densities (500 to 4500 cells/mm² [67]), meaning that at lower densities neurons establish more synapses with each of its neighbors. Since the strength of each multi-synaptic link increases, the number of neighbors necessary for activation (*m* in the QP model) would be reduced by the same factor : $\frac{m}{k}$ remains constant.

QP simulations predict that under these circumstances, the populations are more excitable at lower density (Fig. 27). In experiments however, it was observed that sparse hippocampal cultures (plating density of 450 cells/mm²) burst at a frequency lower than in denser cultures (900 and 4500 cells/mm²)[68], and spontaneous bursting actually disappears completely below a critical density. In cortical networks for instance, the minimal plating density required for the occurrence of spontaneous burst lies between 250 and 500 cells/mm² depending on culture conditions[69].



FIGURE 27. – **Effects of neuronal density with constant total synaptic input.** In each case, the distribution of incoming links follows a Poisson distribution of mean \bar{k} , proportional to the density. The ratio $\frac{m}{\bar{k}}$ is kept at a constant value of $\frac{1}{2}$, which mimics the maintenance of total synaptic afferents[67]. At lower densities (lower \bar{k}), the threshold for burst is smaller.

Now, the absence of spontaneous bursts does not necessarily contradict the prediction of the QP model, that sparse cultures are more excitable than dense cultures. Indeed, it is verified that bursts can be evoked easily in sparse cultures by externally stimulating a single neuron, while even repeated stimulations failed to trigger bursts in denser network[68]. It is therefore more plausible that the absence of spontaneous bursts in sparse cultures is due to the lack of intrinsic generative mechanisms in spite of a lower threshold for burst initiation.

Several mechanisms were proposed to drive spontaneous bursting (random release of neurotransmitters, random firing of neurons, pacemaker neurons, etc...) however none of them is expected to depend on culture density (as they occur punctually). On the other hand, the absence of bursts could be
explained by finite size effects, inherent in growing sparse cultures on limited area. Indeed it was observed that small populations, even when they are dense, do not burst spontaneously[70]. Interestingly, if spontaneous bursts depend on the the number of neurons in the culture and not on their density, it means that the mechanisms of burst initiation are non-local. Recently, a detailed mechanism, remarkably in line with this scenario, has been proposed under the name of noise focusing [71].

Since the plating density (on a fixed surface area) affects both the number of neighbors and the total number of neurons, it is difficult to use density alone to control both the excitability and the spontaneous bursting rate of a population. By changing another independent variable, like the surface on which neurons are seeded, it might however be easy to control each aspect individually.

Alternatively, 2D patterns can be used to reduce the number of accessible neighbors without changing the density of the culture. In a study [72] where neurons where grown of 2D grid-like patterns of different complexity (4, 6 or 8 neighbors), it has been shown that the mean bursting rate in a culture increases as the number of accessible neighbors decreases. Since the total number of neurons is kept constant, the higher bursting rate can only be explained by an increased excitability, which further validates the prediction of QP that a lower connectivity (while maintaining the total number of synapses per neuron) leads to an increased excitability.

We can sum up all these different results and formulate a working hypothesis in which the spontaneous bursting rate of a neuronal population depends on 1) the threshold for burst initiation, which depends itself on the number of neighbors each neuron has, and 2) the level of spontaneous activity, which depends on the total number of neurons in the population.

Although both 1) and 2) can in theory easily be tuned using density, size and micro-patterning of the substrate, we shall not forget that in practice, populations are living components, and that neuronal density and other parameters are critical for survival too. For instance it is well known that sparse cultures, especially in the case of cortical neurons, are more difficult to sustain than denser cultures. More investigation is therefore required to determine not only how to efficiently control the activation function and spontaneous activity of neuronal populations, but also which solutions are compatible with the biology of neurons.

Effects of decay

In the second paper I have studied the effects of decay on the QP model. Decay represents the temporally limited influence of firing synapses on post-synaptic potentials. In the standard model, decay was omitted under the assumption that avalanches happen too fast, in cultured neuronal networks, for decay to have any effect. However, we considered necessary to investigate this hypothesis by actually implementing decay in the QP model. In such implementation, the different signals constituting the potentials of inactive neurons can vanish, at each time-step, with a probability d.

A most fundamental result is that in infinite networks, the activation functions at $m < m_c$ lose their singular threshold-like behaviors and become sigmoids when decay is non-null. For small decay and m values, as it is the case in cultured networks, the sigmoids present however very steep slopes, and finite size network consequently still display threshold like activation function, albeit with higher threshold values.

Now, QP describes the response of a network after a fraction f is activated, but in the model this fraction is activated all at once. If in the absence of decay it does not matter how much time it takes to activate f (because the states of activated neurons is permanent), the temporal profile of activation would become critical in the presence of decay. To understand this we can imagine the extreme scenario in which neurons in f are activated one by one at a rate much lower than the decay time constant. Since each neuron individually can not initiate an avalanche (unless m = 1), and that by the time another neuron is activated the effect of previous activation has vanished, we would always expect at the end of the process that $\phi = f$. Therefore, decay is critical when we do not only consider the size of f but also how fast it is activated. Experimentally, it is quite understandable in the possible differences between prolonged, weak stimulations and short, strong stimulations.

In the particular case of neuronal devices where two populations are connected together by axonal projections, the sharpness of the burst on one side would be determinant for the transmission of the burst to the other side. Indeed, if the activation threshold of the receiving population is bound to a particular time-window, bursts would propagate only if the emitting population sends enough signals during this time window. Therefore the probability of burst transmission could be changed not only by affecting synapses between population, but also the synchronicity of bursts in the emitting population. A recent paper describes how repeated global stimulation in neuronal cultures can increase the sharpness of bursts[73]. Such strategy could therefore be a realistic way to increase the effective communication between units inside neuronal devices.

Effects of inhibitory neurons

The activation function of a neuronal population will of course also depend on the fraction of inhibitory neurons present in the network. The effects of inhibitory neurons were investigated in the frame of the QP model. A surprising result is that inhibitory neurons do not affect qualitatively the percolation in the network. Regarding its mean-field behavior, a network of connectivity k with a fraction of inhibitory neurons f_i is equivalent to a purely excitatory network with a reduced connectivity $\bar{k} [1-2f_i]$. If this prediction only works if excitatory and inhibitory neurons have strictly similar properties besides their contribution to the quorum (+1 and -1 respectively), this might still give us a hint that the presence of inhibitory neurons does not challenge our view of the mechanisms governing the activation functions of neuronal populations as a whole.

However if we additionally planned to exploit the subtle circuitry of neurons inside and between the different populations, inhibitory neurons would become very important. For instance, the order of activation of the neurons, in one population, would be determinant in the transmission of the bursts to a second population, depending on whether the first signals received are excitatory or inhibitory. The first neurons to respond in the receiving population would critically affect the population response as well. The transmission of bursts in neuronal device could therefore depend not only on the total synaptic strength between populations and the sharpness of the burst, but also on the very order of activation of the neurons in each population. I discuss the implications of this in greater details in the last chapter.

Conclusions

As we have seen, the QP model offers a convenient mathematical framework to understand how different parameters, like the connectivity, the leakiness of neurons, and the inhibitory fraction, can affect the behavior of neuronal populations. However we also highlighted some of the limitations of this idealized model. More elaborate simulations, taking into account the spiking dynamics of neurons, the laws governing the number, localization and strength of synapses, and the different forms of plasticity, could help predicting better the behavior of not only neuronal populations but also, in combination with the model presented in the previous chapter, complete neuronal devices.

Interfacing neuronal devices

Introduction

In order to use neuronal devices as computers, an interface allowing us to send them instructions and read the outcome reliably is imperative.

As mentioned previously, extracellular electrodes are often used to read and elicit electrical activity in neuronal cultures. However, even if the embedding of electrodes in custom neuronal devices is possible, it adds a tremendous cost to their fabrication. Furthermore, reading the electrical activity on the different electrodes also requires a costly and heavy instrumentation. I propose instead a fully optical method to interface neuronal cultures, where neurons are stimulated using optogenetics, and recorded though activity dependent fluorescent dyes. The advantage of this interface is also that it can be implemented using relatively standard lab equipment (a microscope).

In a first place, we present a paper demonstrating that such fully optical interface is indeed possible. By applying this method to pairs of connected neuronal populations, we could establish a link between the probability of burst transmission from one side to the other with the physical connectivity between them. This opens a new window on functional connectivity, allowing quantitative studies of synaptic plasticity and learning in neuronal devices.

In the second part, I present the development of a neuronal chip reader that could be used in place of microscopes and fast camera to make high throughput experiments more accessible and user friendly.



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Combining Microfluidics, Optogenetics and Calcium Imaging to Study Neuronal Communication *In Vitro*

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Abstract

In this paper we report the combination of microfluidics, optogenetics and calcium imaging as a cheap and convenient platform to study synaptic communication between neuronal populations *in vitro*. We first show that Calcium Orange indicator is compatible *in vitro* with a commonly used Channelrhodopsine-2 (ChR2) variant, as standard calcium imaging conditions did not alter significantly the activity of transduced cultures of rodent primary neurons. A fast, robust and scalable process for micro-chip fabrication was developed in parallel to build micro-compartmented cultures. Coupling optical fibers to each micro-compartment allowed for the independent control of ChR2 activation in the different populations, we finally show how this platform can be used to evaluate quantitatively the effective connectivity between connected neuronal populations.

Introduction

Behind the apparent simplicity of dissociated neuronal cultures lies an incredibly rich catalogue of behaviors present *in vivo*, including growth and differentiation [1, 2], plasticity [3, 4], electrical activity [5-10] and information processing [11-13]. The culture of primary neurons, extracted from healthy or diseased animals, has thus rapidly become an invaluable tool to understand the nervous system, from the molecular actors of neuronal functions, to the behaviors produced by embodied cultures. Interfacing such cultures is however a necessary step to understand many of these processes. For this purpose, Microelectrodes Arrays (MEAs) have remained the favorite alternative.



Competing Interests: The authors have declared that no competing interests exist.

MEAs have excellent temporal resolution, extended coverage, and their non invasiveness makes them particularly suited for long-term experiments. While originally low, their spatial resolution has significantly improved through recent advances [14]. On the down-side, combining MEAs with micro-compartments, although possible [15-18], is unpractical as it requires alignment steps with the electrodes for each sample, while the costs and technical efforts to set up a MEA platform are substantial and consequently not adapted to non-specialized laboratories. These limitations can however be circumvented using optical methods to excite neu-

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rons and record neuronal activity as an alternative to MEAs. On the one hand, the field of optogenetics has developed alternative methods to control neuronal activity by transducing genes coding for light-gated ion channels into neurons [19-22]. Depending on their nature and the associated ionic flux, photo-activating the channels depolarizes or hyperpolarizes the membrane, and ultimately induces or inhibits action potentials in the modified neurons. Using structured light [23, 24] or LED arrays focused on the specimen [25] allows for spatial resolution superior to those achievable with MEAs, admittedly at the expense of sophisticated optical systems and immobilized samples.

On the other hand, since action potentials in neurons go along with a calcium influx, it is possible to indirectly measure the activity of neurons by calcium imaging using standard fluorescence microscopy [26-30]. Combining these two aspects is appealing, since it opens the route to a contact-less fully optical control and monitoring of neurons activity. However, using light to concurrently stimulate and monitor neuronal activity requires the spectral properties of the stimulating channel and the calcium imaging read-out to be compatible in order to avoid constant background stimulation during imaging. For instance, Channelrhodopsin-2 (ChR2) variants excited with blue light and Fluo-4 are the most popular candidates for optogenetic stimulation and calcium imaging, respectively [31, 32], but the two are not compatible according to the aforementioned criterion. Several ChR2 variants with red-shifted excitation peaks [20] have been developed, but they remain substantially activated by blue light due to their large absorption peaks. Using UV-shifted calcium indicators (typically Fura-2) in combination with ChR2 [33] is not an optimal solution either, due to the harmful consequences of prolonged UV exposure on the cells. Finally, some groups have demonstrated in vivo the possibility to use a standard ChR2 variant in combination with a red-shifted calcium indicator [29, 30].

Besides the stimulation and recording aspects, the experimental toolbox for neuronal cultures has been recently enriched by new nano- and micro-technologies allowing the reconstruction of neuronal pathways under controlled geometry [34-37] and with oriented synaptic connections [38-44], opening new routes for studying cognitive processes in vitro [13, 40, 45, 46]. Geometrical constraints, whether imposed on single neurons or on whole networks, can furthermore facilitate observations in contexts where spatial resolution is a limiting factor.

In this paper, we demonstrate the possibility of combining optogenetics, calcium imaging and microfluidics to study neuronal connectivity. First we report that the Calcium Orange indicator, which spectrum is red-shifted compared to Fluo-4, is a suitable tool to record the activity of neuronal cultures transduced with ChR2 for extended periods of time. Secondly we evaluate the possibility to combine optical fibers and micro-compartmented cultures to increase the spatial resolution and the specificity of stimulation. Finally, we demonstrate the ability of our platform to measure functional connectivity between small neuronal populations connected through different types of micro-channels.

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Materials and Methods

Micro-fabrication

We used PDMS-on-glass chips consisting in micro-wells (2 mm in diameter) in which neurons were seeded, with bundles of micro-channels (Fig 1) connecting them. The low height (3 μ m) of micro-channels was set so as to prevent cell somas from invading them, while their length (500 μ m) allowed neurites to differentiate into axons and connect the different compartments together. While straight micro-channels insured symmetrical connectivity, funnel-shaped channels (15 to 2 μ m) favoured axonal growth in the narrowing direction [42]. This asymmetric design, called "axon-diode", uses a simple geometrical selection: growing axons have a higher probability to enter a wide entrance than a narrow one in the chambers wall. Once in the channel however, they essentially continue to grow with the same speed and efficiency, irrespectively of the small slanting associated with the asymmetry of the channel.

Master molds were created by spin coating a silicon wafer with a first 3 µm thick layer of SU-8 2005 (Microchem) and were then exposed with a first photomask so as to create the axon-selective micro-channels. A second layer of SU-8 3035 was spun at low speed on top of the axon channels and exposed with a second photomask in order to create pillars of 2 mm in diameter and 1 mm tall. Although we don't present them here, we successfully created with this method smaller pillars of 1 and 0.5 mm in diameter. These pillars constitute the templates for the micro-wells. Master molds were replicated in PDMS (Sylgard 184, Dow Corning, 10:1 volume ratio) using two cycles of silanization, casting and curing. The parts to be silanized were first treated with plasma to activate their surfaces (30 s air plasma 600 mTorr at 18W, Harrick Plasma), placed in a petri dish with a drop of silane (1H,1H,2H,2H-Perfluoro-octyl-trichlorosilane, ABCR) deposited on the cover, and incubated 15 min at ambient pressure and temperature. PDMS molds are more resistant to casting-peeling cycles and can be produced easily from the intermediate PDMS counter molds, reducing clean room time and costs tremendously.

The PDMS molds were silanized before the first use and spin coated with fresh PDMS so that the top of pillars from the mold emerge. This step is critical as overflowing the pillars will yield closed micro-wells in the final product. In the event that the micro-wells are closed, they might be opened by simply tearing the PDMS membrane off the wells with clean tweezers. The PDMS parts featuring the wells and micro-channels were assembled on clean glass coverslips (18 mm diameter No.0) using plasma bounding (45 s air plasma 600 mTorr at 18 W, Harrick Plasma). After plasma activation of both coverslip and PDMS, a thin film of Poly-L-Lysine (PLL, Sigma) was created on the coverslip by adding 10 µL of 0.01% PLL, and the PDMS was deposited on the coverslip immediately. Mild pressure insured good contact between PDMS and glass while rejecting the excess of PLL in the wells and micro-channels. Curing 15 min in an oven at 70°C allows the PDMS to attach covalently to the coverslip and the PLL to dry. The microchips were rinsed thoroughly 3 times with double distilled water (DDW) to remove excess PLL, detrimental to neurons. During the rinsing step, it is important to prevent resuspended PLL from adsorbing on the top surface of the PDMS, since this would create unwanted routes for axonal growth. The rinsed chips were finally incubated with plating medium in 12 well plates (1 mL per well) for at least one hour before seeding.

Ethics statement

Animal care and experiments were conducted in accordance with standard ethical European Committee Guidelines on the Care and Use of Laboratory Animals and the guidelines published by the Institutional Animal Care and Use Committee of the Weizmann Institute of



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Fig 1. Design of a simple neuronal device. A- Scheme showing the neuronal device featuring two micro-wells seeded with hippocampal neurons (DIV 15) and connected by an array of axon diodes. The neurons colored in green correspond to neurons that are transduced by ChR2. Neurons colored in purple are not transduced. B- Image of a neuronal device obtained by confocal fluorescence microscopy showing neurons expressing ChR2-YFP (false color).

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Science (IACUC of the Weizmann Institute of Science). Experiments were approved by the IACUC of the Weizmann Institute of Science (WIS application 00420113-2) and were performed in an authorized establishment under the supervision of authorized investigators. Pregnant rats were ordered from Harlan (Rehovot, Israel). Animals were sacrificed the day of arrival using an intraperitoneal injection of pentobarbital followed by cervical dislocation.

Neuronal cell culture

Primary neurons were obtained from rat embryos at stages E19. Dissection and papain dissociation of hippocampi was done according to established protocols[47]. Briefly, brains from embryos were micro-dissected on ice in L-15 medium without phenol red (Life technologies) supplemented with 0.6% glucose and 0.2% gentamycin. Hippocampi were digested in papain solution (papain 100 units, DNAseI 1000 units, L-Cystein 2 mg, NaOH 1M 15 µL, EDTA 50 mM 100 µL, CaCl₂ 100 mM 10 µL, dissection solution 10 mL) at 37°C for 20 minutes. After digestion, the supernatant was carefully removed and replaced with 10 mL of plating medium (MEM without glutamine supplemented with 0.6% glucose, 1% GlutaMAX, 5% Horse Serum, 5% Fetal Calf Serum and 0.1% B27) supplemented with 25 mg of trypsin inhibitor and 25 mg of Bovine Serum Albumine for 5 min to inactivate the papain. Supernatant was removed, replaced with plating medium and tissues were triturated with fired polished pasteur pipettes. The medium covering the microchips was aspirated and the dissociated neurons were seeded in compartment at a density around 5000 neurons/mm². The seeded chips were incubated half an hour in a humidified, 37°C and 5% CO₂ incubator to allow the attachment of neurons to the PLL substrate. Each chip was finally covered by 1 ml of serum-free medium (Neurobasal, B27 4%, GlutaMAX 1% and FCS 1%) and the cultures were placed back into the incubator.

Glial proliferation was stopped 4 days after plating by adding in the medium 20 μ g/mL 5-fluoro-2'-deoxyuridine and 50 μ g/mL uridin (Sigma, Israel). All media contained 50 μ g/mL gentamicin so as to minimise contaminations.

Viral infection

Neurons were transduced with an Adeno-Associated Virus (mixed serotype AAV1-2) containing a pAAV-CaMKIIa-ChR2(E123T/T159C)-P2AE-YFP construct kindly given by the group of Pr. Ofer Yizhar. Due to the low cytotoxicity of AAV, we used a saturating amount of viral particles (extracted from culture supernatant) in accordance to the amounts used in Pr. O. Yizhar lab, yielding a fraction of ChR2-YFP positive neurons of around 70%. Cultures were infected by adding the virus directly to the wells a few hours after seeding. Experiments were performed at least 5 days after infection.

Calcium imaging

The 50 µg of Calcium Orange indicator provided in each tube of the commercial AM Ester (Molecular Probe product) 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 129 mM, KCl 4 mM, MgCl₂ 1 mM, CaCl₂ 2 mM, Glucose 10 mM, HEPES 10 mM) to obtain a loading solution with a final concentration in calcium indicator of 5–10 µM. The culture medium 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 fresh recording buffer, and the cultures were allowed to recover at 37°C for 30 min in the dark before imaging. This step also facilitates hydrolysis of the internalized AM ester precursor into its active form. Images were acquired with a scanning confocal microscope (Leica SP5) and a white-light laser set to 549 nm was used to excite the Calcium Orange. The scanning line was 1

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by 256 pixels, with a pixel size of 4 μ m by 4 μ m. The line was positioned parallel to the axon micro-channels, astride the two populations (S1 Fig). Either end of the line was overlapping its corresponding population by ~ 35 pixels, that is, a 4 μ m by 140 μ m area, over which fluorescence was integrated. The laser was exciting the line in a bidirectional way at a frequency of 8 kHz. The fluorescence was integrated over 8 cycles for each data point, resulting in a final sampling rate of 1 kHz.

Stimulation

Optical stimulations were performed with a custom apparatus consisting of blue (470 nm) Rebel LEDs with 10 mm Square CoolBases (70 lm at 700 mA, LUXEON STAR LEDs). The LEDs were mounted on an aluminum heat-sink with thermal tape. MOSFETs (IRF840) controlled with an Arduino Leonardo board were used to achieve fast modulation of the LED forward currents (from 0 to 700 mA). The LEDs were butt coupled to 0.5 mm diameter PMMA optical fibers with a homemade adaptor keeping the fibers aligned with the LEDs. Home-made spacer and adapter were used to position the other end of each fiber on top of the different neuronal populations, at a distance of approximately 1 mm from the neurons. Since the acceptance half-angle of the PMMA fibers we used is about 26°, the spot receiving the vast majority of the light was about 1.5 mm in diameter. Each 0.5 mm fiber delivered about 3 mW of light in this configuration, as measured with a laser power meter (Ophir, Israel).

Results and Discussion

In vitro compatibility of optogenetic stimulation with calcium imaging

To be able to combine optogenetic stimulations of neurons with calcium imaging, we needed to select a red-shifted calcium dye and check if the excitation of this dye did not trigger neuronal activity in population of neurons expressing ChR2. The calcium dye we have selected, Calcium Orange indicator from Molecular Probe, presents a maximum of excitation at 549 nm (Fig 2(B)). The minimum light intensity at 549 nm (obtained with a white-light laser) required to yield a sufficient signal to noise ratio was first established on non transduced cultures as a starting point for experiments.

The effect of increasing intensities of 549 nm light on ChR2 was then investigated on cultures expressing ChR2-E123T/T159C. Recorded bursting rates were normalized by the bursting rate observed under minimal light on the same culture, so as to exclude inter culture variability in term of basal activity. The results showed that activity was largely unaffected by light at 549 nm. Increasing the intensity of excitation light by 400% (compared to the reference level) yielded no significant increase (paired t-test: p > 0.25, n = 9) in the frequency of bursts (Fig 2(A)). This shows either that despite a broad absorption peak, ChR2 is not activated, or that neurons compensate for ChR2 currents through some homeostatic mechanism. However, the fact that we could induce bursts reproducibly using pulses of light at 470nm (see below) during calcium imaging shows that most of the ChR2 channels are in a responsive, thus unactivated state under such conditions.

Based on the theoretical figures of merit, other ChR2 variants (such as H134R presented in Fig 2(B)) have been considered as they could tolerate even higher levels of light at 549 nm without background stimulation of neurons (Fig 2(B)). However the maximal light intensity used to excite calcium indicators is still limited by other factors, like bleaching or phototoxicity, so that in practice the advantage of using other variants might not be as great.

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Short stimulations ignite bursting events in transduced cultures

We characterized the response of transduced neurons to brief pulses of light (from 1 ms to 1024 ms), at 470 nm and for a dose of about 3 mW/mm² (Fig 3). Stimulations were performed every 5 seconds, a rate low enough to avoid possible short term plasticity effects but also high enough to reduce the probability of occurrence of spontaneous bursts (a inter-burst interval of 5 to 10 seconds is common at 13 DIV). Note that in the case of Fig 3 where the temporal resolution was not the most critical aspect of the experiment, we binned the data by chunks of 50 ms in order to yield less noisy curves.

It is important to notice that two mechanisms are involved in the activation of neurons in these responses. The first one is the direct activation by light (via ChR2) which thus applies only to transduced neurons; the second one is an indirect activation by synaptic transmission and it applies to all neurons. To better characterize the contribution of each of these two mechanisms, we did experiments in the presence and absence of CNQX.

CNQX is an antagonist of AMPAR, a ionotropic transmembrane receptor for glutamate that mediates most excitatory transmission at synapses. In consequence, under saturating concentration of CNQX, excitatory synaptic transmission is blocked and the observed response only represents the fraction of neurons that is directly activated by light.

In the absence of CNQX we observed that each stimulation elicited a bursting event, the amplitude of which, as recorded using calcium imaging, was largely independent from the stimulus duration ($\underline{Fig 3(A)}$). Notice that in spite of our fairly high stimulation rate, a burst does occur spontaneously just before one of the stimulations. However it appears that another burst is evoked at the actual stimulation time. It seems therefore that the stimulations are strong and override any possible natural post-burst refractory period.



Fig 2. Compatibility of ChR2 and Calcium Orange. A- Quantification of background stimulation in ChR2(ET/TC) transduced cultures during calcium imaging with Calcium Orange at DIV12. The abscissa indicates the intensity of excitation light (549 nm) used during imaging relative to the recording conditions used in the rest of the experiments (1x); 2x is twice that level and 4x is 4 times that level. The different bursting rates at 2x and 4x were normalized by the bursting rates recorded at 1x for each culture so as to remove inter-culture variability for initial bursting rates. For information purpose, average absolute bursting rates 1x was 5.9 ± 2.2 bursts per minute (n = 9 cultures). B- Absorption spectra of two ChR2 variants, including the ChR2(ET/TC) used in this study, as well as those of the commonly used Fluo-4 and its red-shifted counterpart Calcium Orange indicator. The figures of merit of each combination are indicated in % of maximal absorption.

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After the addition of 10 μ M CNQX (determined experimentally as the lowest saturating concentration), the response to the shortest stimulus was significantly reduced compared to normal condition, and increased gradually with the stimulus duration (Fig 3(B)) in contrast to the former case. This shows that under normal conditions, the amount of neurons originally activated by light is actually dependent on stimulus duration although synaptic activation eventually takes over and leads after a percolation process to a same final state.

Surprisingly, responses to longer stimulations were consistently higher in the presence of CNQX. This paradoxal finding could be explained by the simple hypothesis that inhibitory neurons do not express ChR2. This hypothesis is supported by the observed mutually exclusive marking of GAD2-positive (inhibitory) and ChR2 positive neurons (<u>S2 Fig</u>), in line with former work [<u>48</u>] reporting that the CaMKII promoter (used in our experiment to express ChR2) is significantly less active in inhibitory neurons than in excitatory neurons. During stimulation in the presence of CNQX, the inhibitory neurons would remain silent because their sole excitation pathway is blocked. While the inhibitory neurons (10–20% of neurons in culture) would not contribute to the collective response anymore, the rest of the culture, uninhibited, would be able to respond more strongly upon ChR2 activation.

The possibility that the first neurons to respond might be only excitatory would change the modality of recruitment of neurons in the burst. It therefore raises the more general question of whether individual neurons experience evoked bursts differently from endogenous ones based on the timing of activation of their pre and post-synaptic partners. Although our data does not allow us to conclude, other groups have developed complementary methods to monitor fast propagation of neuronal activity in small neuronal populations[45, 46]. Such methods



Fig 3. Optogenetic induction of bursting events. A- Typical responses of a transduced culture (DIV 13) to different stimulation durations (1, 2, 4, 8, 16, 32, 64, 128, 256, 512 and 1024 ms). Vertical blue bars represent stimulation times and durations. No fluorescence data was recorded during the stimulations as the photodiode, recording directly the stimulated spot, was saturated. B- Same experiment as in A after addition of 10 µM CNQX (AMPA receptor antagonist).

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could help us determine whether evoked activity is fundamentally different from spontaneous one, and understand the implications of stimulations regarding possible plasticity and adaptation effects in cultures.

Locally induced bursts propagate through axon channels

In order to study neuronal functional connectivity, we used a system in which two populations (contained in 2 mm diameter chambers) were connected by symmetrical micro channels, but only one population was expressing ChR2 ($\underline{Fig} 4(\underline{A})$). To independently control the illumination of the two populations, we developed a system based on optical fibers in which each fiber is coupled to an LED on one end and a neuronal population on the other end, and in which each LED comes with an independent control circuit.

Stimulating the transduced side with 5 ms pulses reproducibly evoked bursts in the transduced population, as well as in the non transduced population (Fig 4(B)). As non transduced neurons do not directly respond to light, we concluded that functional synapses between the two populations must have formed, allowing activity to propagate from the transduced side to the non transduced side. This transmission of activity is in accordance with results from Peyrin et al. who already demonstrated that neuronal activity can propagate through axons grown in micro-channels [42].

In contrast, neither side produced a bursting event when the non-transduced population was stimulated. In some cases like presented on $\underline{Fig 4(C)}$ we observed a minimal response in both populations probably due to the activation of a subnetwork too small to ignite actual bursting events. Since in all cases, light diffusing from the other side was not able to ignite a burst in the transduced population, we concluded that there is no light-induced crosstalk between the two chambers in our system.

Axon diodes produce asymmetrical functional connectivity

When both populations are transduced, the absence of crosstalk previously demonstrated guarantees that post-stimulus activity evoked reproducibly in the non stimulated population is due to synaptic transmission only. We thus applied our system to a pair of transduced populations in order to evaluate transmission independently in each direction, by initiating a burst on either side and measuring the response in the opposite side over 30 repetitions, so as to average out any non specific response (like spontaneous activity occurring by chance in the peri-stimulus time window). Thanks to the short data collection window allowed by line-scanning (see methods), we could record the response to stimulations in each chamber with a sub millisecond resolution. We could thus evaluate the delay of transmission from one population to the other, by maximizing their normalized cross correlation coefficient. Chips with straight channels produced a strong and symmetrical connectivity, where the delay of transmission was systematically smaller than 5 ms (Fig.4(B)) suggesting a high reciprocal connectivity between the two populations.

In a second approach we used narrowing micro-channels called "axon-diodes", already known to orient axonal growth and synaptic transmission [42] (see methods for details), and we observed a strong functional polarity of the neuronal connectivity. In one third of all cases (n = 15 pairs), activity exclusively propagated in the forward (or narrowing) direction of the axon channels (Fig 5(B)). In the other cases, reverse transmission also occurred, albeit with a significantly greater delay (paired t-test: p < 0.005, n = 10) with a relative increase comprised between 23% and 211% (Fig 5(C)). We interpreted this increased delay as the time necessary to integrate enough signals, from a reduced number of axons, to ignite a burst. The fact that reverse transmission could occur is not totally surprising for two reasons. The relative fraction of

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Fig 4. Local stimulation and burst propagation (DIV15). A- Picture of a symmetrical two-compartments device with only one side transduced (left). Green and red channels are for ChR2-YFP and Calcium Orange indicator, respectively. This dual marking reveals that 70% of cells are expressing ChR2 inside the transduced compartment. B and C- Averaged response after stimulation of the transduced (B) and non-transduced (C) compartments. The solid colour lines correspond to the peristimulus fluorescence signal averaged over 30 stimulations (0.1 Hz in alternation) while the filled surfaces indicate associated standard deviations. Vertical dashed lines indicate stimulations. Diagrams are presented for clarity, with matching line and population colors. Green neurons are transduced neurons.

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Fig 5. Burst transmission in asymmetrical networks (DIV15). A—Explanatory diagrams for graphs presented on the left and right column respectively, with the same color code as in Fig 4. B—Typical example of a device showing unidirectional transmission. C—Results from a device where bursts also propagate in the reverse direction, albeit with a greater delay. Addition of 5 μ M CNQX to the latter device yields the typical unidirectional transmission (D). Delays obtained by normalized cross correlation.

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axons growing in the reverse direction is not negligible; it has been previously estimated to be around 3% [42]. Secondly the minimal number of activated neurons to ignite a burst is apparently small according to the results presented in Fig 3; only a few axons would be necessary to relay a burst.

Interestingly, reducing the overall excitatory neurotransmission by adding onto these cultures an under-saturating concentration of 5 μ M CNQX restored an absolute unidirectional transmission (Fig 5(D)). Our interpretation is that CNQX increases the threshold of burst initiation to the point that the reverse connectivity becomes insufficient while the forward connectivity, originally stronger, can still carry enough signals to trigger a burst in the output population. These threshold effects and their link to synaptic strength can be explained by the quorum percolation model[49]. It is clear that the presence or lack thereof of burst transmission on the one hand, and the delay in burst transmission on the other hand, are two sides of the same coin, namely, functional connectivity.

These experiments show that the functional connectivity yielded by axon diodes is highly polarized and confirm the previous results of Peyrin et al. [42]. The results also suggest how delicate it is to maintain an absolute unidirectional transmission when the residual reverse connectivity is amplified by percolation effects. Luckily in case of bidirectional transmission, delay measurements still provide information about differential connectivity. We thus believe that this functional approach offers a new and easy way to evaluate quantitatively and with great sensitivity the synaptic connectivity between neuronal populations, complementing direct synaptic imaging and electrophysiology.

Conclusion

In this paper, we have described an experimental setup, combining optogenetics, calcium imaging and microfluidics, as a tool to measure functional connectivity between different populations of neurons in reconstituted networks.

The optical recording and stimulation method used in this setup has by itself significant advantages over micro-electrode arrays. Besides alleviating the need for micro-electrodes fabrication technologies and tedious alignement of electrodes with micro-patterned substrates, the method allows for a fully non-contact system where all the sophistication of the measurement is retained in the non-disposable optical components of the instrument, and all the elements in contact with neurons are disposable, reducing the costs and facilitating sample preparation. In systems like the presented micro-chips, where a high spatial resolution is not necessary, a temporal resolution comparable to that of MEAs could be achieved at a far lesser cost, using photodiodes instead of a scanner or an ultra fast camera.

The prospect of measuring effective synaptic strength without heavy and expensive electrophysiology techniques is very promising for large scale studies on synaptic plasticity, memory and their importance in neurodegenerative diseases.

With ongoing developments aiming at increasing the throughput of the experiments, by recording and stimulating multiple chips at once, a large number of parameters such as neuronal type, culture conditions, network topology, or stimulation patterns could be systematically examined to get deeper insights into fundamental aspects of neuronal networks behavior.

Supporting Information

S1 Fig. Calcium imaging acquisition process. A. The scanning line of the confocal microscope was positioned following the dashed line so that it covered both populations in roughly equal proportions. B. Resulting imaging data are x-t pictures, from which we extracted the fluorescence traces presented in Fig 4 and Fig 5. The dashed lines delimit the two regions over

which fluorescence was integrated. A stimulation initiated on the right side is visible in this recording, and appears as a blue stripe.

(PNG)

S2 Fig. GAD2 inhibitory neurons do not express ChR2. The image shows a primary culture of neurons extracted from GAD2-Cre × ROSA:loxp-stop-loxp:tdTomato embryos (JAX 010802 and 007909 respectively). Only the GAD2-positive inhibitory neurons expressing the Cre recombinase can excise the floxed stop codon and express tdTomato (in red). It appears clearly that ChR2-YFP (green), controlled by the CamKIIa promoter, is not expressed in GAD2-positive inhibitory neurons.

(PNG)

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Author Contributions

Conceived and designed the experiments: MV RR SB EM. Performed the experiments: RR MV NS. Analyzed the data: RR. Contributed reagents/materials/analysis tools: SB EM LM SD PM JMP JLV. Wrote the paper: RR MV SD SB EM JMP JLV.

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A low cost and scalable chip reader

In the previous paper we have seen that a fully optical interface for neuronal devices is possible, however the way it was implemented in this paper had some important drawbacks. Firstly the optogenetic stimulations were done using optical fibers which required tedious positioning before each experiment. Secondly the scanning line of the confocal microscope we used could only record the activity of two populations at once, which represents a strong bottleneck for the throughput of the experiments. Finally microscopes, while often part of the common lab equipment, still constitute heavy instruments that are not adapted for massively parallel, large scale studies. Finally the size of the microscope itself prevents the use of neuronal devices as embedded systems (in robots for instance).

In order to overcome these limitations and still take advantage of the optical interface, I designed and build a dedicated neuronal chip reader that could palliate to the aforementioned inconveniences, by being at the same time accessible to any laboratory, easy to use, and allowing higher throughputs.

General design

Accessibility mostly means using low cost and easily available parts. The most expensive piece of equipment in the previous implementation was clearly the confocal microscope. The major drop in price would therefore come from an alternative fluorescence detection system. Indeed if we consider the neuronal populations to be the most elementary units inside our neuronal devices, there is no need to resolve spatially the details within the different populations. The total fluorescence emanating from each population can instead be collected individually, using independent photodiodes instead of a single high-speed cameras. The amplification of the photodiode signal was done using a home made lock-in amplifier which also only requires low cost components (available from Radiospares). The circuit board was cheap and compact enough to have one for each detection channel.

In order to further lower the costs, we propose an optical configuration (Fig. 28) based on common parts (available from Thorlabs and Omega Optical) and custom 3D printed parts. Using 3D printing to fit exactly the optical components also guarantees robustness of assembly and removes fine tuning steps for the user. Note that in this configuration, the optogenetic stimulations are done from the same side as fluorescence excitation, so that no tedious alignment step is required and the interfacing of neuronal devices is further facilitated.

As the task that required the largest amount of work was the detection of low levels of fluorescence, the developments of the amplification system were first carried out on a simple optical prototype comprising a single observation spot. In order to increase the flexibility and the throughput of the experiments,



we started a first scale-up by incorporating in the reader 6 independent recording/stimulating channels, allowing to investigate for instance 3 pairs of connected populations at once, or a single network of 6 populations.

FIGURE 28. – Schematic representation of the optical setup. This schematic represents a single recording/excitation channel.

Optics

As we wanted a user friendly system that is robust and does not require fine tuning, we printed the optical case to accommodate perfectly the lenses, mirrors and filters. However because no adjustment is possible afterwards it was necessary to determine accurately the best optical configuration. To determine the optimal positioning of the different components without spending to much time and effort, we developed a dedicated ray tracing algorithm to simulate and evaluate different configurations.

The ray tracing algorithm was based on the implementation of the Snell-Descartes law at diopters. This low level engine allowed to simulate accurately the behavior of commercial optical components (lenses, glass slides, dichroic mirrors, etc...) for which the dimensions and material properties were known, including possible optical aberration.

The positions and orientations of the different optical components were extracted from successful simulations and used to design the 3D printed parts.

The parts were created using the FreeCAD software. An example of optical ray tracing simulation superimposed on the model of the 3D printed case is presented in figure 29.

Compartmented neuronal device



FIGURE 29. – **Ray tracing simulations.** Results obtained for 3 recording/excitation channels under the configuration presented in Fig. 28. The lenses (in white) are 2 cm in diameter.

Printing was done using a commercial 3D printer as well as a personal 3D printer (mini-Kossel), both based on fused deposition modeling technology, allowing a resolution of $\approx 200 \ \mu\text{m}$. Other types of 3D printers based for instance on stereophotolithography can be used for finer resolutions. The inside of the optical block was painted with deep black optical paint so as to remove stray light and autofluorescence of the printed plastic parts.

Electronics

Lock-in amplifier

The lock-in amplifier is a detection system that allows to recover a signal buried in a very noisy background. The general principle is to give the signal of interest a precise frequency signature and subsequently filter out all other frequencies. Since noise is spread over a very broad spectrum of frequency, a narrow band pass filtering removes an enormous amount of noise. In order to faithfully transcribe the temporal evolution of the signal, it is however critical for the modulation period to be significantly smaller than the duration of the events we want to detect. As we want to detect burst, the rise time of which is a few tens of milliseconds, the fluorescence level was in our case modulated by making the fluorescence excitation LEDs blink at 20 kHz.

At the core of the lock-in amplifier is a modulator/demodulator (AD630 modem) which performs the filtering step by multiplying the noisy signal with

To photodiode array

a periodic reference signal of the same frequency as the true signal. There are several ways to understand how this actually lead to the elimination of noise. The first one is mathematical : the product of two periodic signals of different frequency has a time average of 0. On the other hand when the two signals have the same frequency, the average is non-zero (unless they are ± 90 out of phase) and maximal when they are in phase. Therefore if the periodic reference signal given to the modem has the same frequency than the modulated signal, and that the phase difference between them can be tuned to 0, the output will have a non zero average which will not depend on noise levels. A more intuitive way to understand how noise is filtered out is to see the reference signal as a command to alternatively change the sign of the noisy signal. If the true signal and the reference are in phase, then the output values for "noise + true signal" (led on) and "noise alone" (led off) will have different sign, which allows basically a simple subtraction of the noise from the noisy signal. In order for the frequencies of the reference and the true signal to be locked together, we controlled the LEDs and the reference signal with the same square wave generator. However because of the non-zero response time of the photodiode, the signal controlling the LEDs has to be advanced so that the true signal carried by the photodiode output and the reference signal have the same phase. The way this is achieved is explained in the next subsection.



FIGURE 30. – **Schematic for the lock-in amplifier.** A more detailed schematic of the circuit can be found in the appendices.

The complete lock-in amplifier circuit (Fig. 30) includes firstly an instrumentation amplifier (AD620). The output of the instrumentation amplifier is then multiplied to a reference square signal with the same frequency and phase as the true signal. The high frequency component of the modem output is filtered out and the DC component is amplified through a last amplification stage with tunable gain and offset. The PCB were designed using KiCAD and ordered from Eurocircuits. The boards were assembled using a soldering iron but reflow soldering is also possible to solder surface mounted components (Fig. 31).



FIGURE 31. – **Lock-in amplifier after assembly.** The potentiometers to tune the gain and offset are visible. Technical specifications of the PCBs can be found in the appendices.

Square wave generator

The signal is generated by charging and discharging a capacitor by alternatively connecting it to V+ or V- via 2 MOSFETs (Fig. 32). When it is connected to V+, the capacitor is charging and the output of the generator is high. When the capacitor voltage U_c reaches the threshold $+U_{t1}$, it becomes connected to V-, starts to discharge towards the new threshold $-U_{t1}$ and the output is low. When U_c reaches $-U_{t1}$, the capacitor is connected back to V+, the threshold switches back to $+U_{t1}$ and a new cycle begins. The charging and discharging time of an ideal RC circuit are supposed to be identical but we used a potentiometer of total resistance *R* to tune the duty cycle precisely to 50%, which is important for the functioning of the lock-in amplifier.

The period $(\frac{1}{f})$ of the signal is defined as the sum of the charging time and the discharging time, which depend on the RC constant but also on the separation of two thresholds $+U_{t1}$ and $-U_{t1}$. There is therefore another potentiometer to tune the value of $\pm U_{t1}$ in order to modulate the frequency of the generated signal. In an ideal circuit, the period is equal to $RC\ln(\frac{1+\alpha_1}{1-\alpha_1})$, where α_1 is the relative resistance of the ground side compared to the total resistance of the potentiometer ($0 \le \alpha_1 \le 1$). When α_1 is not to close to 1, the period is $\approx 2RC\alpha_1$. Finally, we duplicated this signal using a second reference $U_{t2} = \alpha_2 U_{t1}$ with $0 \le \alpha_2 \le 1$. Because the U_c reaches the value $\pm U_{t2}$ before $\pm U_{t1}$, the second comparator produces a square wave signal which is advanced compared to the reference that will be fed to the lock-in amplifier. The advance in an ideal circuit with 50% duty cycle is equal to $\frac{RC}{2} \ln(\frac{1-\alpha_1\alpha_2}{1-\alpha_1})$. When α_1 is far from 1, this advance is $\approx \frac{1}{2}RC\alpha_1(1-\alpha_2)$. Therefore the relative advance is $\approx \frac{1-\alpha_2}{4}$, meaning that the maximum phase difference attainable with this circuit is $\frac{\pi}{2}$.



FIGURE 32. - Schematic for the square wave generator.

LED driver

A stable reference voltage V_{ref} is created by the reverse voltage of a zener diode (~2 V independently of V+/V-) and a voltage divisor which allows to tune V_{ref} between 0 V and 2 V. V_{ref} is fed to the non-inverting input of an operational amplifier.

The LEDs in series are powered by a separate voltage generator, and the current through the LEDs i_{LEDs} is regulated by a power MOSFET controlled by the output of the operational amplifier. The current going through the LEDs is converted into voltage across the resistance R and fed to the inverting input of the operational amplifier.

Due to this feedback, the voltages at each of the amplifier's inputs are maintained equal, therefore $i_{LEDs} = \frac{V_{ref}}{R}$. In addition, V_{ref} can be shorted to 0 V through a N-c c cMOSFET. This allows to modulate the LEDs current easily using the wave generator.

Results and discussion

After several months of optimization, the lock-in amplifier could detect bursts with a reasonable signal to noise ratio (Fig. 34) while using a relatively low power LED for fluorescence excitation (Nichia LED NSPB300B, 70 mA @ 3



FIGURE 33. - Schematic for the LED driver.

V). This result was however obtained with cultures loaded with Fluo-4 indicator and not with Calcium Orange, which is, as we saw in the paper, necessary to combine calcium imaging with optogenetic stimulations. While the relative increase in fluorescence upon calcium binding is significantly smaller in the case of Calcium Orange, it has to be put in perspective with the higher level of fluorescence excitation it is possible to achieve with it. Two reasons concur to this. Firstly, the light used to excite Calcium Orange (549 nm) is less harmful to cells than shorter wavelengths (470 nm for Fluo-4) so the intensity of light can be increased. Secondly, Calcium Orange is significantly less sensitive to photobleaching (the destruction of fluorophore by light) than Fluo-4 [74]. By exciting Calcium Orange at a higher level, fluorescence emission could be increased significantly. Since the the noise in the system is mostly due to electronics, increasing fluorescence emission levels should also lead to a better SNR.

The fact that detection of burst is possible with this technology remains a striking result. Currently the complete six populations neuronal chip reader is under construction but there is no reason to doubt at this point that it will work as expected.

Conclusion

In this chapter we have demonstrated the possibility to interface neuronal devices, grown in microfluidic chips, using a convenient fully optical and contact-less technology. We also make the proof of concept that such interface can be implemented with inexpensive and readily available parts, and undertook to build an all-in-one chip reader allowing to read and control 6 neuronal populations independently. The following chapter illustrates an



FIGURE 34. – **Raw output of the lock-in amplifier imaging an hippocampal culture at DIV11.** The typical profile of bursts, with a fast rise time and a slow fall time, is clearly identifiable. The bursting rate was identical to the one observed later under the microscope, confirming that the events detected with the home made reader were most probably not artifacts.

application where such device would have been a life-changer, namely the characterization of synaptic plasticity in cultures. The difficulties encountered along numerous and long experiments were in fact the main reason behind the development of a dedicated neuronal chip reader. We believe that such cheap, accessible and user friendly instrument could democratize the use of neuronal devices in various other teams, might they be theoreticians taking their first steps in the realm of *in vitro* artificial intelligence, neurophysiologists looking for complementary methods to study synaptic plasticity *in vitro*, or pharmaceutical start-ups eager to target neurological disorders.



 $\ensuremath{\mathsf{Figure}}$ 35. – 6-populations neuronal chip reader currently under construction.

Teaching neuronal devices : looking for Synaptic Plasticity

Introduction

So far, we have seen that neuronal devices can be fabricated and interfaced reliably, and that several strategies are theoretically possible to program them. Within the proposed framework, computations in neuronal devices are expressed by the probability of bursting in the different output populations after instructions were given, under the form of optogenetic stimulations, to the different input populations.

We have discussed in the second chapter how different elements could affect the transmission of bursts in a network of populations, including the synchronicity and order of firing of neurons in the different populations. However, targeting these parameters to develop learning strategies is not consistent with the idea that in our case populations are indivisible, black-box units.

Instead the most relevant parameter to program neuronal devices is the strength of synapses *between* the different populations. We have seen in chapter I that the initial connectivity can be tuned using different kinds of topographical guides for axons. However we are interested in the possibility to change the strength of synapses after the growth of the network has taken place, so as to refine existing functions or learn new ones. Such changes are therefore related to the processes of synaptic plasticity.

As discussed in the introduction, synaptic plasticity involves several mechanisms and pathways. Among them, Spike-Timing-Dependent Plasticity (STDP) links the amplitude and sign of synaptic change with an easily controllable parameter; the spiking times of the pre- and post-synaptic neurons. STDP is particularly attractive to us, because in our case, the pre- and post-synaptic neurons can be in two different populations and therefore be stimulated independently.

In particular, the order of spikes between neurons can determine if the synapses are reinforced (potentiation) or weakened (depression). In general, STDP describes long lasting effects, where potentiation and depression are still observed 30 min after the induction. In this case we talk about Long Term Potentiation (LTP) or Long Term Depression (LTD). In the canonical STDP rule [75, 76, 77] observed between excitatory neurons, LTP occurs when the post synaptic neuron fires repeatedly right after the pre-synaptic neuron (in a 5-50 ms time window), while LTD occurs when neurons fire in the reverse order. However, numerous other STDP rules have been reported [26, 78], depending on the neuronal types constituting the pairs, and the system in which they were observed.

The primary goal of this part was therefore to investigate how the possible STDP rules, acting between individual neurons, merge into global Burst-Timing-Dependent Plasticity (BTDP). Direct scaling of STDP protocols to population pairs yielded transient changes in the way bursts propagated in either direction. Unfortunately, we will see that none of these effects were maintained over long enough periods of time to confirm the presence of LTD or LTP in the cultures. In order to determine the origin of the problem, we investigated the activation of the NMDAR-CaMKII LTP pathway (Fig. 36) in cultures using our optogenetic induction protocols as well as chemical inductions as positive controls. As we shall see, it appeared that this pathway was mostly not affected by any of these protocols.



FIGURE 36. – **LTP pathway.** Left : During normal synaptic transmission, glutamate (Glu) is released from the presynaptic bouton and acts on both AMPA receptors (AMPARs) and NMDA receptors (NMDARs). However, Na⁺ flows only through the AMPA receptor, because Mg^{2+} blocks the channel of NMDAR. Depolarization of the postsynaptic cell relieves the Mg^{2+} block, allowing Na⁺ and Ca²⁺ to flow into the dendritic spine. The resultant rise in Ca²⁺ within the dendritic spine is the critical trigger for LTP. Right : Simplified model for the expression of LTP. An increase in Ca²⁺ within the dendritic spine binds to calmodulin (CaM) to activate CaMKII, which undergoes auto-phosphorylation, thus maintaining its activity after Ca²⁺ returns to basal levels. CaMKII phosphorylates AMPA receptors (AMPARs) already present in the synaptic plasma membrane, thus increasing their single-channel conductance. CaMKII is also postulated to influence the subsynaptic localization of AMPA receptors such that more AMPA receptors are delivered to the synaptic plasma membrane. Figure reproduced from [79]

Material and Methods

Fabrication of microfluidic chips, cell culture, optogenetics and calcium imaging were carried out like in the previous chapter.

Burst-Timing-Dependent Plasticity protocols

The BTDP experiments were directly inspired by historical STDP induction protocols found in the literature [75, 76, 77]. The induction phase consisted in the repeated optogenetic stimulation of two connected populations (either through straight symmetrical channels or axon diodes) for a short time (typically 1 min) at a frequency *f* (from 1 Hz to 50 Hz) and with time shift of Δt (from 0 to ±50 ms). The *effective* connectivity before and after induction was evaluated by stimulating each unit alternatively, every 10 seconds for 5 minutes (30 repetitions), and recording the response in both of them via calcium imaging, as explained in the third chapter. During recording, we either imaged the whole field comprising the two populations (slow recording, 6 Hz) or a single line overlapping partially each population (fast recording, 1 kHz) as explained in the third chapter. This is specified in the result section.

ANQX experiments

ANQX is an irreversible blocker of AMPA receptors. It binds covalently to AMPARs when exposed to UV light. ANQX is not commercially available; synthesis of ANQX was done by Dr. Veronica Frydman from the organic synthesis service of the Weizmann Institute, following the protocols in [80]. The resulting ANQX powder was diluted in DMSO at 100 mM (24.8 mg/mL) and the stock solution was kept at -20°C. ANQX was diluted down to 100 μ M in external medium and applied to neurons. Cultures where subsequently exposed to UV light (360 nm) with a UV transilluminator, then washed with fresh EM.

Chemical Induction

The protocol for chemical induction was adapted from [81]. It consists in depolarizing the cells by applying transiently high K⁺ solutions. It involves two solutions :

- 1. Solution 1 : External Medium (EM : NaCl 129 mM, KCl 4 mM, MgCl₂ 1 mM, CaCl₂ 2 mM, Glucose 10 mM, HEPES 10 mM, pH 7.4) supplemented with bicuculline 100 μ M and tetrodotoxin 0.5 μ M (Sigma-Aldrich).
- 2. Solution 2 : A high K⁺ variant of solution 1 (KCl 90 mM , NaCl adjusted to 43 mM to maintain osmolarity).

Cultures were taken out from the incubator and coverslips were incubated at RT in solution 1 for 5 minutes, then dipped 3 times in solution 2 for 1s with an interval of 10s. After the induction, the coverslips were incubated back 15 minutes in solution 1, and fixated for immunostaining.

Preconditioning

The preconditioning and induction protocols where taken from [82]. Briefly, APV at 50 μ M was added to hippocampal neurons for a minimum of 2 days before the experiment. Cells were transferred to External Medium supplemented with APV at 50 μ M. The conditioning itself involved replacing the EM by a Conditioning Medium (CM) containing 200 μ M glycine, 3 mM Ca, 0 M APV and 0 M MgCl₂ (other concentrations identical to EM) for 5 min. After 5min, The CM was washed out and replaced with the standard APV-containing EM.

Immunostaining

Immunostaining was done following the protocol in [83]. Briefly, coverslips were fixated in a 4% PFA/Sucrose solution for 10 minutes, permeabilized with Triton X-100 0.1% in PBS for 10 minutes, then blocked for 1 hours with BSA 5% in PBS. The primary antibodies (Abcam) were diluted in 1% BSA PBS and applied to the cultures, in a humidified chamber, overnight at 4°C. Neurons were rinsed 3 times in PBS, then Alexa Fluor secondary antibodies (Life Technologies), diluted in BSA 1% were applied for 1 hour at room temperature. The coverslips were rinsed again 3 times in PBS, mounted on a glass slide using ProLong anti-fade (Life Technologies) and sealed with nail varnish.

Results

Pairing protocols yielded no long-term effects.

Due to the low throughput of the experiments, only 2 different BTDP induction protocols could be investigated. The first one consisted in 1 min long inductions at 1 Hz with a Δt of +10 ms. The induction was done on symmetrical devices (straight channels between the two populations). In this set of experiments, the time resolution was very low (170 ms), as we first imagined that changes in connectivity would only affect the probability of burst transmission (which is visible at low temporal resolution), and not the delay in burst transmission as well. By comparing the post stimulus activity profiles before and after the induction (Fig. 37), we did not observe any long-lasting change.

However, we did almost systematically observe changes in the bursts profiles immediately after induction. These effects are difficult to explain solely by some kind of passive mechanisms such as synaptic fatigue, as we observed in some cases that bursts amplitudes and durations actually increased in the receiving population (Fig. 37 B).

The second induction protocol consisted in 1 min inductions at 10 Hz with a Δt of +20 ms or -20 ms in asymmetrical devices (axone diodes). This set of experiment was carried out at a much finer temporal resolution (see methods).



FIGURE 37. – Pairing protocol between two symmetrically connected populations. Post-stimuli profile before and after induction (60 stimulations at a 1 Hz and Δt =10 ms, blue first), averaged over 30 repetitions. The filled surfaces represent standard deviation. The observed direction of propagation is indicated on the left. The induction led only to non lasting effects (dashed lines are guides for the eye). A : In most cases, bursts appeared slightly smaller immediately after induction. B : In a few cases, the output population responded by larger and/or longer burst shortly after induction. Note that all these changes have been reversed back to the original situation within 30 minutes after induction.

Once again, we observed only short term effects. However it became clear that these changes were following some patterns.



FIGURE 38. – **LTP protocol between two population.** Post-stimuli profile before and after induction (600 stimulations at a 10 Hz and Δt =+**20 ms**). Black arrow indicates transient potentiation. The black curve represents the baseline before induction.

When traditional LTP protocols were implemented to reinforce the forward connectivity (Fig. 38), we observed that the communication in the forward direction was actually slightly impaired, with a slightly smaller response amplitude in the output population (the small change in delay transmission is not significant). More surprising, the reverse connectivity, although initially too weak to allow propagation of burst in the reverse direction, was potentiated to the point that such propagation became possible (see black arrow).

Reversely, when traditional LTD protocols were applied to reduce the connectivity in the forward direction (Fig. 39), we actually observed poten-



tiation of the forward connectivity with a significantly larger response in the output population, while the delay of burst transmission was increased and the response amplitude decrease in the reverse direction.

FIGURE 39. – **LTD protocol between two population.** Post-stimuli profile before and after induction (600 stimulations at a 1 Hz and Δt =-**20 ms**). Black arrow indicates transient potentiation. The black curve represents the baseline before induction.

Although we conducted this experiment in only 4 different pairs, we observed consistent transient potentiation in the expected LTD direction and depression in the expected LTP direction. This result is striking for two reasons. Firstly it means that populations are indeed sensible to the timing of events occurring in a narrow temporal window (10 ms time scale). Secondly, the way
BTDP emerges from STDP rules acting between individual neurons turns out to be nontrivial, as we observed patterns diametrically opposed to traditional STDP rules.

So far, we have no idea how these effects are affected by neuronal types, culture conditions or induction protocols; such studies will be necessary in the future to determine how to induce BTDP reliably. However, given the size of the parameter space on the one hand, and all the possible experimental vagaries on the other hand, it is clear that such a comprehensive study on plasticity cannot be carried out with the low throughput setup used until now. This motivated the conception of a more appropriate tool, namely the dedicated neuronal chip reader presented in the previous chapter.

In the mean-time, the fact that the observed changes were not maintained for long periods of time revealed that the LTP and LTD pathways were not working as expected. In order to determine the origin of the problem, we monitored several sub-mechanisms occurring in particular along the LTP pathway. We will focus first on the the recruitment of new AMPA receptors from intracellular reserves, which constitute the final step in the chain of events leading to LTP.

Optogenetic induction does not lead to recruitment of new AMPA receptor.

In order to follow the recruitment of new AMPARs at synapses, a classical strategy is to irreversibly block receptors already present in the membrane, and monitor how synaptic communication is recovered after blockade. Such strategy was implemented in brain slices successfully to determine if LTP induce reinsertion of new AMPARs in the membrane of synapses [84]. We conducted equivalent experiments in transfected cultures, albeit at the scale of whole populations.

After blockade of the AMPA receptors, we induced LTP with theta-burst stimulation (TBS), which consist of bursts of four pulses at 100 Hz with an interval of 200 ms, for one minute. Spontaneous bursting activity before and after induction was compared (Fig. 40). It appeared that the LTP induction did not lead to any recovery of the bursting rate, meaning that some upstream mechanism was impaired. We therefore continued our investigation targeting the CaMKII kinase which plays a pivotal role in the transduction of the LTP pathway.

Optogenetic induction does not activate CaMKII in transfected cultures.

Although it was previously reported that CaMKII is phosphorylated and translocates into dendritic spines upon optogenetic induction of LTP[85], nei-



FIGURE 40. – **Recruitment of new AMPARs after LTP induction.** Spontaneous bursting was monitored in a culture before blockade of AMPAR (ANQX binds irreversibly to AMPAR when exposed to UV light), and after blockade + LTP.

ther the levels of phospho-CaMKII, nor its localization were significantly affected by TBS inductions in our experiments (Fig. 41).

It was however observed that the expression of CaMKII and ChRII-YFP was anti-correlated. This was surprising given that the ChR-YFP transgene is expressed under the control of CaMKII promoter, and that they should there-fore be strongly co-expressed. Such anti-correlation could be explained by the

integration of multiple copies of the ChR2-YFP transgene into easily accessible regions of the chromatin, monopolizing the pool of transcription factors targeted to the natural CaMKII promotor. Whatever the cause of this interference might be, it was clear that it was possibly causing the LTP problem. It was therefore necessary to carry out additional experiments in non-transfected cultures.

No induction

After induction



FIGURE 41. – **Immunostaining of phospho-CaMKII.** In green is ChR2-YFP. In Blue is phospho-CaMKII. In red is Synapsin1. Scale-bar is 50 μ m.

Chemical induction does not activate CaMKII in non transfected cultures.

In order to induce LTP in non-transfected cultures, we used a chemical induction protocol [81], based on the transient application of high extracellular K^+ concentrations to neuronal cultures. Indeed as the cell membrane is, at rest, mostly permeable to K^+ , the membrane potential is determined by the reversal potential of K^+ , E_{K^+} . Under normal saline concentration (solution 1 in methods) E_{K^+} sits around -80 mV. In solution 2, extracellular K^+ concentration is increased to match intracellular concentration, and $E_{K^+} \approx 0$ mV. TTX and bicuculine were added to blocks spikes, inhibitory synaptic currents, respectively. Each dip in solution 2 was therefore expected to produces a step increase in the membrane potentials of neurons, from -70 mV to ≈ 0 mV.

At this point, we also started to investigate the effects of neuronal density, as we thought that dense and very active cultures might have already saturating levels of phospho-CaMKII. While similar experiments carried by other groups produced strong effects on phospho-CaMKII levels and localization (see for instance [86]), chemical induction did not lead to significant activation of CaMKII in either sparse or dense cultures (Fig. 42). We came to the conclusion



FIGURE 42. – **Chemical induction of LTP.** In green is phospho-CaMKII. In red is Synapsin1. Scale-bar is 50 μ m.

that CamKII activation, and all downstream events, were simply not working in our cultures. We therefore finally targeted the most upstream event in the LTP pathway, namely the entry of calcium through NMDA receptors.

Increased NMDAR currents do not enhance CaMKII activation.

In order to reduce the possible bottleneck occurring at this stage, we maximized NMDAR activation by cultivating neurons in a preconditioning medium, which normally leads to the over expression of NMDA receptors through a compensating mechanism[87]. The induction itself was carried out using a medium which allowed maximal activation of NMDAR and calcium currents (no blocker, no magnesium, high glycine and high calcium extracellular concentrations). We also investigated on the same batch the effects of neuronal density, as well as transfection, in order to have a comprehensive set of data. However, once again, no significant effects were observed in any of the different conditions (Fig. 43). Similar preconditioning/induction experiments were carried out on cultures during continuous calcium imaging. Although such conditioning led to increased burst amplitude in other groups[88], no such effect was observed.

Discussion

LTP is normally divided in two phases, induction and maintenance. CaM-KII is mostly involved in the induction of LTP and its maintenance over the first hour following induction, while other kinases such as PKA and PKC are involved in longer-term maintenance[89]. It is therefore surprising that we could not observe any sign of CaMKII activation in our cultures, while it is supposedly responsible for the observed short term effects we observed in BTDP induction experiments.

We can propose several hypothesis so as to reconcile those two elements. The first hypothesis would consist in the very fast activation and deactivation of CaMKII after induction. Indeed we observed that the short term effects were not extending beyond 30 minutes (Fig. 37), but they might actually last for a much shorter amount of time. Given that CaMKII immunostaining experiments were done 15 minutes after induction, we might have missed transient effects. However this hypothesis alone does not explain why no AMPAR where recovered after induction of LTP.

Results could also be explained under the hypothesis that CaMKII is basally active even in low density cultures. Indeed, it seems that phospho-CaMKII is already localized at synapses (see colocalization of Synapsin1 and phospho-CaMKII in Fig. 42) and cell somas even before induction, while such compartmentalization was only observed after LTP in other groups[86]. Under basal over-activation, the internal pools of AMPAR would probably be scarce so that cultures can not recover from AMPAR blockade. Finally increasing NM-DAR currents would have no effect under such hypothesis, because of CaMKII saturation. However this hypothesis would mean that short term effects are actually CaMKII independent. More investigation on NMDAR-CaMKII and other LTP pathways, including the ones involving the kinases PKA and PKC, will be necessary to understand the origin of the observed short term effects.

Regarding the possible basal over-activation of CaMKII that would prevent true LTP and LTD to take place, we can hypothesize that it is due to high levels of spontaneous activity. In line with this hypothesis, we can note that contrarily to the group of Menachem Segal, who observed population-level LTP in isolated groups of less than a hundred neurons, we used relatively large populations comprising about ten thousand neurons. As we discussed in the second



FIGURE 43. – **CaMKII activation 15 minutes after induction of preconditioned cultures.** In green is ChR2-YFP. In Blue is phospho-CaMKII. In red is Synapsin1. Each panel show 4 different fields of view in order to capture the results variability. N. B. : The top left panel might not show representative results because the coverslip was dropped during mounting.

chapter, small populations are spontaneously bursting much less, if bursting at all, compared to larger populations. Continuous activity of large populations would therefore keep CaMKII pathway activated constantly, impeding its proper operation during induction protocols. Even in the case where the STDP pathway would not affected by spontaneous activity, bio-realistic simulations show that spontaneous bursting can erase induced changes (preliminary results). It goes without saying that investigating the effects of populations sizes and more generally spontaneous activity, experimentally, but also by the mean of simulations, is currently of primordial interest to understand population-level plasticity.

Conclusion

In this chapter we investigated plasticity in neuronal devices as a possible strategy to teach them new functions. We showed that potentiation and depression of the connectivity between two populations could be achieved using stimulations inspired from STDP protocols, but that those effects were not sustained over long periods of time. This seems to be attributable to the NMDAR-CaMKII pathway, which remained unaffected by a set of control experiments that should normally strongly activate it. More control experiments are necessary, in particular electrophysiology experiments, in order to determine if the problem actually arises from the STDP pathways in single neurons as it is hypothesized, or from the scaling of those pathways to large populations. In either case, as we do not have a clear explanation of the absence of long-term plasticity, more systematic examination of the other LTP pathways as well as the different culture parameters, in particular the size of populations, will be required. The exploration of such a vast parameter space will become possible in the near future, through the development of the dedicated parallel chip reader presented in the previous chapter.

General conclusion

In this thesis, I proposed a new kind of neuronal device, based on population networks. We followed its development, from the very conceptual idea behind its architecture, inspired by research in artificial intelligence and experimental limitation found in other systems, to its experimental implementation and final evaluation.

Along the way, we have developed tools to facilitate their use. Among these we can mention firstly a theoretical model to predict axonal growth inside the PDMS chips, physical substrate of the neuronal device. A fully optical protocol to interface neuronal devices, based on calcium imaging and optogenetics, was proposed as an alternative to electrodes, so as to reduce micro-fabrication costs. In order to make experiments easier and more accessible, I additionally conceived a dedicated chip reader so as to eventually use neuronal devices as plug-and-play modules.

We used these tools to investigate plasticity in neuronal devices, on which we based the possibility, for neuronal device, to learn new functions. The results of such investigation were mitigated. On the one hand, we could not imprint new "memories" durably into our neuronal devices. However we did see short-term effects after a special induction protocol (Burst-Timing-Dependent Plasticity) that were remarkably consistent. Provided that such changes could eventually be maintained, the BTDP paradigm could lead to the development of efficient strategies to program neuronal devices.

We can already imagine the kind of experiments and protocols that could be carried out in the future.

Further investigations in 1-to-1 devices would of course be necessary to increase our understanding of BTDP rules between neuronal populations, and how it is affected by a large set of environmental variables. However 1-to-1 devices are in practice not that interesting to perform any computation other than the simple transmission of a signal. On the contrary, in branched systems, we could observe additionally interesting non-linearly separable computations. Such computations are easily implementable with a few excitatory and inhibitory neurons, and it is well possible that the appropriate "hidden" circuitry, within and between populations, can be reinforce through simple plasticity mechanisms.

The 2-to-1 devices were originally thought of to this purpose. Two input units are connected to the same output unit through a directed "Y" junction that also spatially randomize the axonal projections to produce an homogeneous initial connectivity (Fig. 44).



FIGURE 44. – Schematic representation of a 2-to-1 device (top view)

In 2-to-1 devices, it is meaningful to study, the possibility of computing non-linearly separable functions. For instance the ability to teach a XOR function can be explored in such device. Although the overall landscape of BTDP parameters to explore is vast, we can for a start rationalize the design of such protocols; as soon as we have more than one incoming connection for a particular output unit, we can differentiate two types of dual (input-output) stimulations :

- In disjoint stimulations, each of the two possible input-output couples is affected independently from the other, through temporal isolation.
- In joint stimulations, the two input-output couples are stimulated simultaneously.

As before, each dual stimulation is characterized by a Δt which represents the delay between input and output stimulation. In the case of symmetric functions like XOR, each disjoint dual stimulations would have the same Δt_d . The joint stimulation will be characterized by a single Δt_j since the two couples are stimulated simultaneously. We can now define a rather compact way of defining the teaching protocols as combinations of one joint and one disjoint dual stimulation (Fig. 45), that are repeated for a given amount of time. For each (Δt_d , Δt_j) couple we can measure at the end the activity in the output unit after stimulating the two inputs either jointly or disjointly.

If the response after joint stimulation of the two inputs is less than after each disjoint stimulations, we would consider we have successfully taught the XOR function to the device and that non-linear functions can be learned by networks of small neuronal populations. If such pilot experiments turn out successful, they would definitely open the door to the development of more



FIGURE 45. – Schematic representation of teaching protocols, with positive Δt_d and negative Δt_j

complex neuronal devices and to a range of new studies and applications we can barely imagine.

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Appendices



Scaling of direction change with step size.

In this small appendix, we are interested to know how the distribution of direction changes, occurring during the addition of new segments in axonal growth, scales with the size of the steps. To understand this, we can ask more specifically what segment length and angle distribution can approximate the growth process occurring over *n* steps of length *d* and with angles $\alpha_i \rightarrow \mathcal{N}(0, \sigma)$. We can understand from the top illustration that the equivalent angle α_{eq} is equal to arctan $\frac{H}{L}$ and d_{eq} to $\sqrt{H^2 + L^2}$. Under ballistic conditions (small σ and small *n*), H <<< L so that $\alpha_{eq} \approx \frac{H}{L}$ and $d_{eq} \approx L$. From the bottom illustration, it is evident that $L = \sum_{i=1}^{n} d \cos(\sum_{j=1}^{i} \alpha_i)$ and $H = \sum_{i=1}^{n} d \sin(\sum_{j=1}^{i} \alpha_i)$. Since the values for α_i are small, and for small *n*, we can apply sin $x \approx x$ and $\cos x \approx 1$. We obtain $L = \sum_{i=1}^{n} d = nd$ and $H = \sum_{i=1}^{n} d \sum_{j=1}^{i} \alpha_i = \sum_{i=1}^{n} d(n+1-i)\alpha_i$. The probability density for α_{eq} is therefore $\frac{\sum_{i=1}^{n} (n+1-i)\alpha_i}{n}$.

$$E(\alpha_{eq}) \approx \frac{\sum_{i=1}^{n} (n+1-i)E(\alpha_i)}{n} = 0$$

$$V(\alpha_{eq}) \approx \frac{\sum_{i=1}^{n} (n+1-i)^2 V(\alpha_i)}{n^2}$$

$$V(\alpha_{eq}) \approx \frac{\sum_{i=1}^{n} j^2 \sigma^2}{n^2}$$

$$V(\alpha_{eq}) \approx \frac{\frac{n(n+1)(2n+1)}{6}\sigma^2}{n^2}$$

$$V(\alpha_{eq}) \approx (\frac{n}{3} + \frac{1}{2} + \frac{1}{6n})\sigma^2$$

If we suppose that α_{eq} follows a normal distribution, then this distribution is $\mathcal{N}(0, \sigma \sqrt{\frac{n}{3} + \frac{1}{2} + \frac{1}{6n}})$. Consequently when we scale *d* by a factor *n*, we should scale σ by a factor $\sqrt{\frac{n}{3} + \frac{1}{2} + \frac{1}{6n}}$.



Detailed schematic of the lock-in amplifier.

Specifications of the lock-in amplifier PCB.

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PCB Passport Order Number: E690685							
PCB name : Small lock-in amplifier Purchase reference : Quantity packed : 5 Article reference : Packing date : 05-02-2015 Project reference : Producer : Eurocircuits Aachen GmbH							
Top view of the PCB	Bottom view of the PCB						
C C C C C C C C C C C C C C C C C C C							
Click on the image to see larger view							
Mechanical layer							

Mechanical layer								
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		Symbol	END.dia (mm)	Tooldia (*0.01mm)	number of Holes	spec.desc. Nibb(N) mill(M)	NPTH	
		0	0.15	25	22	0		
		D	0.80	90	6	0		
		٥	1.00	110	12	0		1



PCB Passport Order Number: E690685

Production flow of outer layers							
Production Step	End of the process	Materials/Measured values	Tolerances/Result				
Start of production	03-02-2015	NP155(FR) 1,55 mm 18/18 um	Pass				
Drilling	03-02-2015	Smallest finished hole size: 0,15 mm	Pass				
Outer layer imaging	03-02-2015	Type of dry resist foil: Kolon PK-1650 http://fratar.com.ar	Pass				
Outer layer galvano	04-02-2015	Value of copper thickness in the holes: 20-28 um	Pass (20-80 um)				
Solderresist coating	04-02-2015	Probimer: Haerter 65/7168 Harz 65/7096-1	Pass				
Plating	05-02-2015	Leadfree HASL	Pass				
Electrical test	05-02-2015		Pass				
Mechanical finishing		Outline milling	Pass (+/-0,2 mm)				
Final inspection	05-02-2015		Pass				