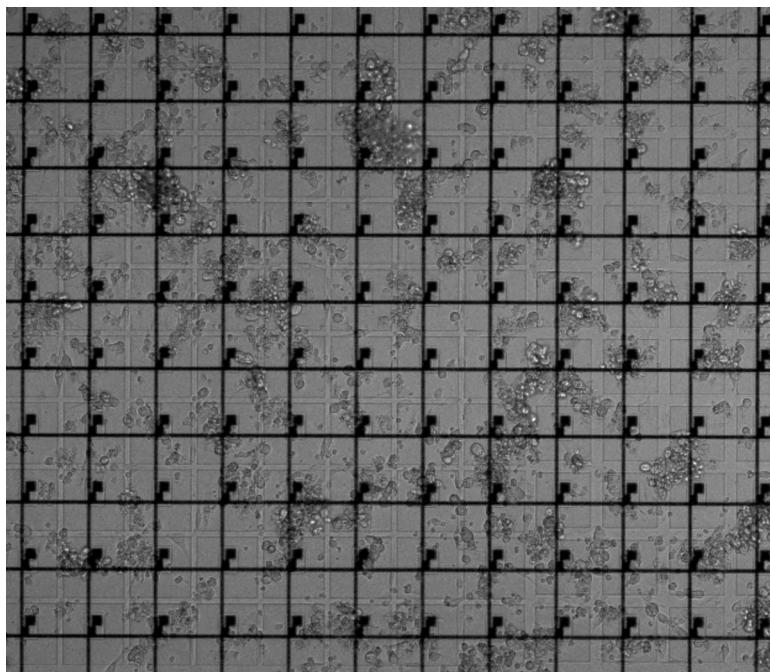


TN09 – Research Internship Report

**Study of the effect of
norepinephrine adrenergic stimulation on
the electrical and mechanical activity of *in vitro* cardiomyocyte
cell culture using a TFT multimodal platform**



26/08/2024 – 14/02/2025

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Acronyms Glossary

CNRS: Centre National de la Recherche Scientifique

CVD: Cardiovascular Disease

DC: Direct Current

DMEM: Darbeco Modified Eagle Medium

DMSO: DiMethyl SulphOxide

IIS: Institute of Industrial Sciences

ITO: Indium-Tin Oxide

LIMMS: Laboratory for Integrated Micro-Mechatronic Systems

MEA: Microelectrode Array

MEMS: Micro-Electro-Mechanical Systems

NEMS: Nano-Electro-Mechanical Systems

NCJ: Neuro-Cardiac Junction

NE/ NA: Norepinephrine / Noradrenaline

NPY: Neuropeptide Y

PBS: Phosphate-Buffered Saline

PCB: Printed Circuit Board

PDMS: Poly-Di-Methyl-Siloxane

TFT: Thin-Film Transistor

Glossary

Darboco Modified Eagle Medium: Widely used synthetic medium for adhesive cell culture.

Digitiser: A device that receives, records and processes analogue data, in this case, electrophysiology signals.

JAPASTIM: Control card developed by the lab.

Medium: Liquid containing growth factor, nutrients, proteins, and antibodies for cell culture.

Micro-Electro-Mechanical Systems and Nano-Electro-Mechanical Systems: a type of micro- to nano-scale miniaturised electro-mechanical elements made with micro-fabrication processes. They can be composed of micro/nano-sensors, micro/nano-actuators, micro/nano-electronics, micro/nanostructures [4] and are mainly used in biomedicine, communication, information technology, environmental monitoring, and automotive industry fields.

Microfluidics: Microfluidics are defined in terms of microlitre volumes or micrometre dimensions of channels. Microfluidics are used in separations, cell analysis, and microreactors, to illustrate a few applications. [5]

Multiplexer: A device that combines several independent inputs into a single output (composite signal).

Phosphate-Buffered Saline: Isotonic buffer used for washing the cells and suspension dilution.

Poly-Di-Methyl-Siloxane: Organic silicone-based polymer widely used for biomedical and microfluidic applications due to its biocompatibility, transparency, and elastic properties. [20]

Thin-film transistor: A transistor fabricated by deposition of thin films on a substrate. This technology is widely used for LCD displays. [21]



Introduction

Cardiovascular diseases represent a major cause of mortality on a global scale with environmental factors and deteriorating living conditions contributing to this burden. These diseases frequently involve a complex interplay between the heart and the brain, forming a physiological axis between the heart and the brain, commonly referred to as the “brain-heart axis”. This axis plays a pivotal role in maintaining organ homeostasis. Disruption in this pathway can result in severe conditions affecting both organs. For instance, chronic stress is frequently implicated in the onset of stress-induced arrhythmias and, the mechanical function and structure of the heart can be affected by strokes, aneurysms, and spinal cord injuries [7]. Conversely, several cardiac diseases can also impact brain function, with partial obstruction of the aortic arch, for instance, leading to a decrease in cerebral blood flow, resulting in cognitive impairments and inflammation [16].

Despite their critical importance in human health, the junction between neuronal and heart cells remains poorly understood, with traditional *in vivo* models, while providing valuable insight into brain-heart interaction, but limited by the complexity of the environment, making it difficult to isolate a specific mechanism, and differing from human physiology. In this context, biohybrid *in vitro* platforms offer a new experimental framework, allowing a more detailed study of cellular and molecular interactions between the heart and brain.

During my internship at the Institute of Industrial Sciences (IIS), University of Tokyo, I specialised in the development of a protocol, utilising an *in vitro* model to investigate how neurotransmitters such as norepinephrine modulate cardiomyocytes behaviour on the electrophysiological, optical and overall scale. Utilising microfluidic technology to create a controlled neurotransmitter-release system in the *in vitro* cell culture alongside thin-film transistor (TFT) technology developed by the lab to monitor cardiomyocyte activity has enabled the achievement of multimodal recordings of cellular response to neurotransmitter stimuli. This work contributes to the growing body of knowledge on the brain-heart axis, and the development of such an *in vitro* model is a crucial step toward future application in drug screening and the study of heart-brain-related diseases.

Key words: Biohybrid platform, TFT array, Electrophysiology, Brain-heart axis, Microfluidics

I. Professional context

I.1. The IIS in an international context

“Institute of Industrial Science (IIS), the University of Tokyo (Figure 1), is a research institute [...] mainly located in Komaba, Meguro-ku, Tokyo. Each of more than 120 faculty members – professors, associate professors and lecturers – has his/her own laboratory and more than 1,000 researchers come to IIS from inside and outside of Japan. They are carrying out various research – from fundamental research to applications – which opens future life.

The research field of IIS is very multidisciplinary and covers almost all the areas of engineering disciplines from micro and nano scales such as quantum levels to large scales such as the global level and space [...].

The IIS promotes international academic exchanges by conducting joint studies with foreign researchers and educational activities for international students. [...] The idea of appointing foreign members to the faculty and sending young Japanese researchers to other countries is encouraged.” [1] (Figure 1)



Figure 1: The IIS Komaba Campus - Institut des Sciences Industrielles à Komaba [a]

I.2. The LIMMS : an alliance between the IIS and CNRS

The LIMMS, Laboratory for Integrated Micro-Mechatronic Systems is a CNRS International Research Laboratory on **MEMS and NEMS** (Micro- and Nano-Electro-Mechanical Systems), jointly operated by the CNRS (INSIS, Institute for Engineering and Systems Science) in France and the IIS in Japan. Since its creation in 1995, LIMMS has hosted more than 175 researchers from France and Europe and covers fields such as BioMEMS. At the moment, the LIMMS is hosting 17 researchers (Figure 2). [2]

The LIMMS research field covers three main axis : Energy, Quantum and Molecular Technologies and Bio (Micro-Nano Systems)

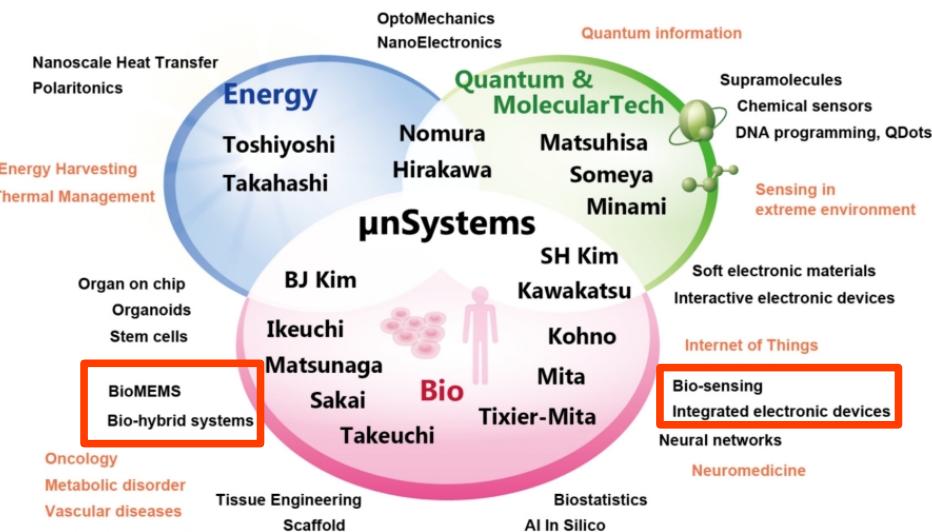


Figure 2: The LIMMS Research fields and researchers - Les chercheurs et domaines de recherche du LIMMS [2]

I.3. The Tixier-Mita Lab

PhD. Agnès Tixier-Mita is currently Associate Professor in the laboratory of Prof. Hiroshi Toshiyoshi, who was recently appointed as the director of the IIS (Figure 3). “Her research interest is to interface with biological cells by means of electrical tools, to sense them, and to interact with them.” [3]

The lab specialises in developing multi modal platforms for biomedical investigations, integrating electronics, microfluidics, and various sensors (electrical, optical, etc.). These platforms are primarily used for *in vitro* multicell culture, enabling external multi modal sensing, such as electrical stimulation to mimic *in vivo* interactions, optical observation, and chemical experiments guided by microfluidic channels. The goal of such platforms is to advance the understanding of unresolved biomedical questions, particularly the interaction between the heart and brain, known as the brain-heart axis.

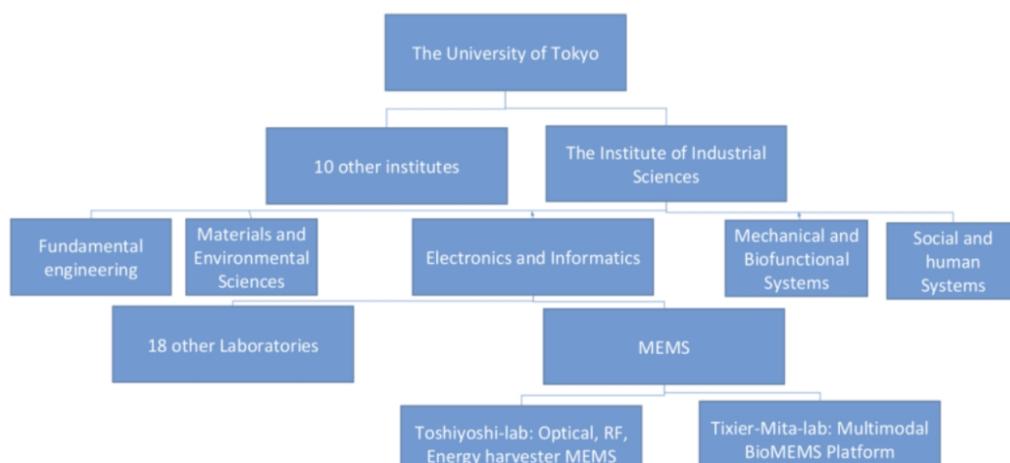


Figure 3: Organisation of IIS laboratories and fields of research - Organisation des laboratoires de de l'IIS et domaines de recherche

I.4. Role within the Tixier-Mita lab

My mission in the Tixier-Mita lab takes place in a larger project focusing on the development of a multi-sensing platform for the study of the brain-heart axis *in vitro*. This project encompasses a wide range of disciplines, with numerous other students contributing to or having contributed to its enhancement (Figure 4). The primary objective of my work is to devise a protocol and conduct some experiments with neurotransmitters (norepinephrine) on cardiomyocytes with the aim of acquiring optical, electrical, and chemical data. The objective of this work is twofold: firstly, to enhance the comprehension of the behaviour of cardiomyocytes *in vitro*, and secondly, to extend the range of applications of the TFT platforms developed in the laboratory. Ultimately, this study is to provide support for the subsequent stage of the laboratory's research which is the development of a device for co-culturing neurones and cardiomyocytes that is compatible with TFT devices in addition to the improvement of knowledge about the neuro-cardiac junction.

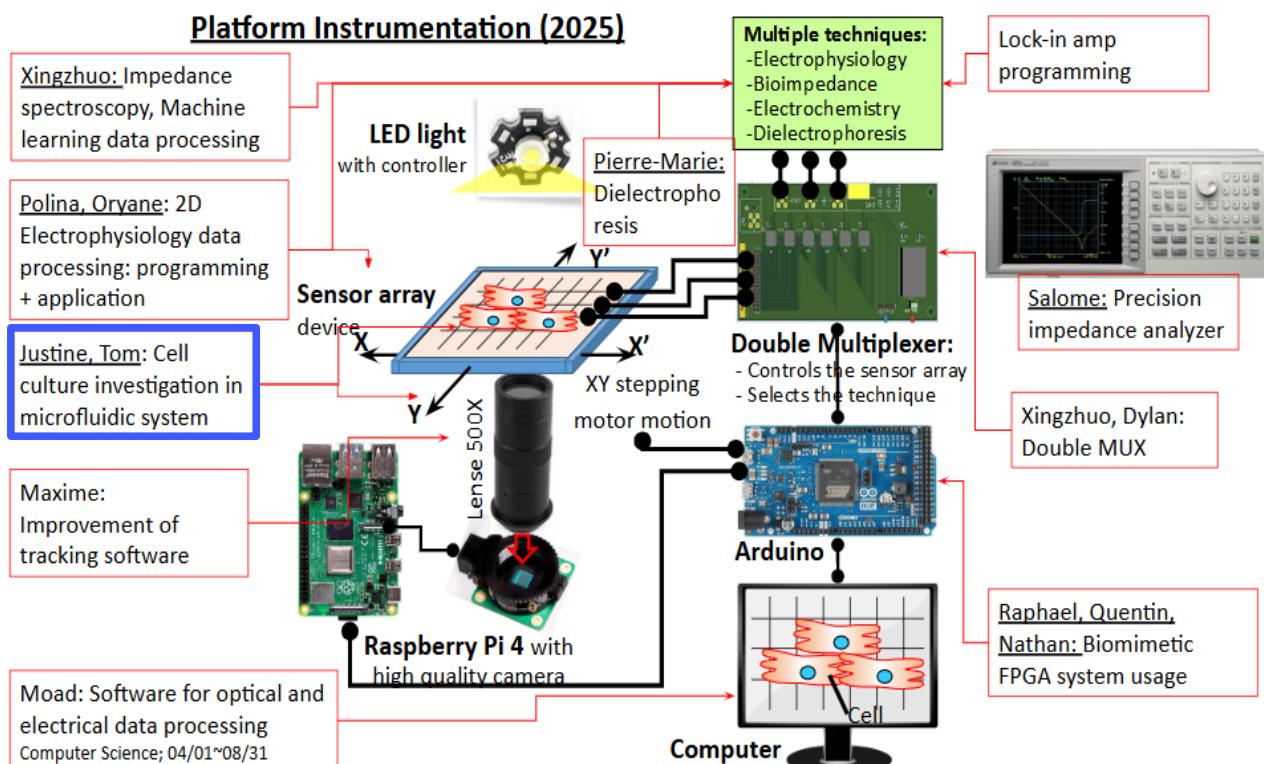


Figure 4: Scheme of the organization of work in the Tixier-Mita lab and tasks associated with each intern, master student and PhD during 2024-2025 – Schéma de l'organisation du Tixier-Mita Lab et des tâches associées à chaque stagiaire, étudiant de master et doctorant au cours de l'année 2024 -2025

I was also given the freedom to take part in other aspects of the project concerning the platform, such as the development of a microfluidic chip for co-culture of cardiomyocytes and neurones.

II. Scientific context

II.1. State-of-the-art

The primary objective of this internship is to deepen our understanding of the behaviour of cardiomyocyte in the presence of neurotransmitters, with the ultimate goal of enhancing our knowledge of the interaction between the brain and the heart.

II.1.a. The cardiovascular system and the heart role in human physiology

The primary function of the cardiovascular system, comprising the heart and blood vessels, is to facilitate the transportation of substances to the body's cells such as nutrients absorbed by the epithelium, oxygen captured in pulmonary alveoli. It is also responsible of the removal of cellular waste to the excretory organs. In addition to its role as a transport route for substances linked to cellular metabolism, the cardiovascular system also plays a role in cellular communication via hormones. The heart functions as a mechanical pump that propels the blood throughout the vascular network. The heart is made of **two pumps**, the right heart and the left heart, separated by a septum. Each side is characterised by two distinct chambers, an **atrium** that receives the blood and a **ventricle** that distributes it into the blood vessels [6] (Figure 5). Non-oxygenated blood is directed to the right atrium, and consequently, the ventricle. The right ventricle contracts to drive the blood to the lungs for re-oxygenation. The oxygenated blood enters the left atrium and thus the left ventricle, and is distributed throughout the entire body and organs.

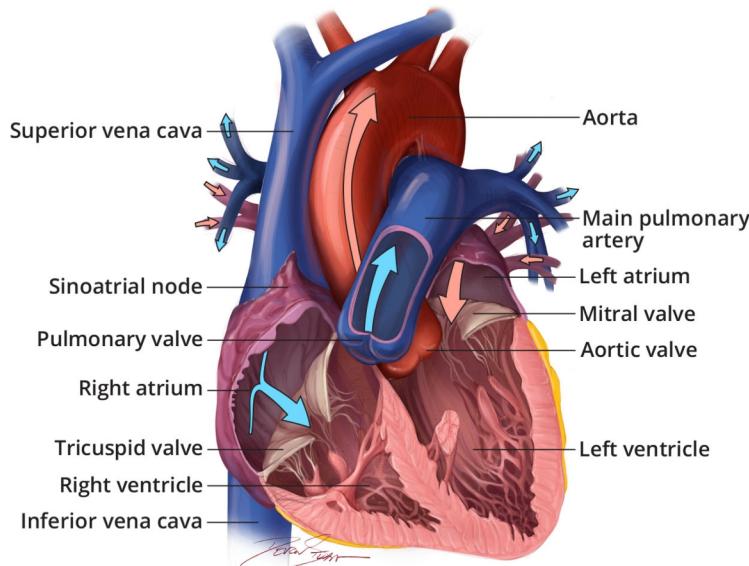


Figure 5: Anatomy of the Heart - Anatomie du cœur [f]

The heart is therefore a critical organ with the slightest malfunction likely to cause a major problem, including death. Alteration of the heart beat can have major consequences in a person's quality of life and life expectancy.

II.1.b. The cardiomyocyte and the brain-heart axis

Cardiomyocytes are a specific type of single-nucleated pacemaker cell found in the heart tissue. These cells are capable of spontaneous production of action potentials, a phenomenon known as auto-rhythmicity. These cells are responsible for the heart's contractile function, whereby they depolarise gradually until they reach a threshold value, which then triggers the action potential (Figure 6), occurring at a rate of approximately 100 contractions per minute (theoretical rate). The slow depolarisation is the result of a process referred to as **calcium-induced calcium release**¹, whereby extracellular calcium entering the cell induces the release of calcium from the sarcoplasmic reticulum.

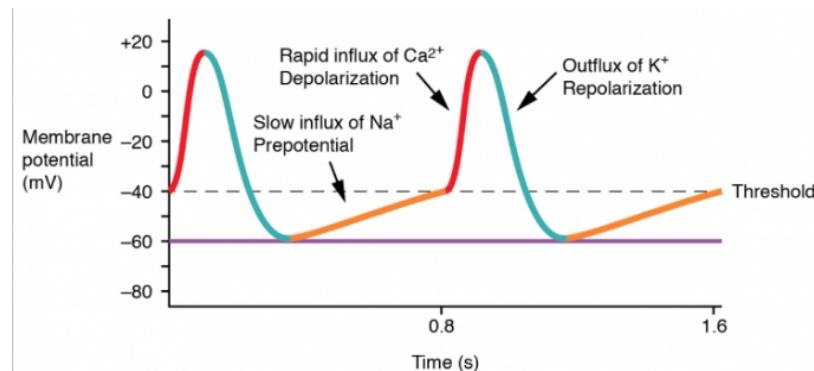


Figure 6: Theoretical evolution of the action potential of a cardiomyocyte cell - Evolution théorique du potentiel d'action d'un cardiomyocite [g]

Cardiomyocytes are made up of myofibrils, allowing the cells to deform with great amplitude during contraction. The majority of cardiomyocytes are concentrated in the sinoatrial node (Figure 4), located in the right atrium of the heart. This is the rhythmogenic centre of the heart, with action potentials almost instantly propagated to all atrial cells through gap junctions located in the intercalated discs; cardiomyocytes are therefore electrically coupled (Figure 7).

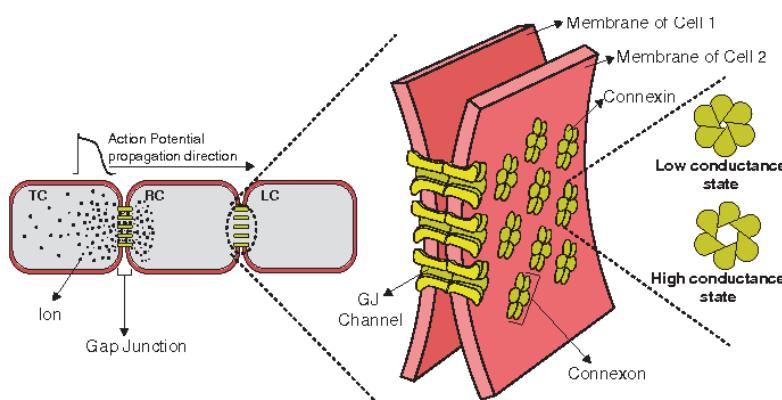


Figure 7: Gap junctions illustration - Illustration de jonctions communicantes [b]

¹ Calcium-Induced Calcium Release (CICR) is a mechanism in which Ca^{2+} entry in a cell (by calcium channel) stimulates more Ca^{2+} liberation by the sarcoplasmic reticulum. It amplifies the calcium signal, essential for cardiac cell contraction.

A salient important property of these cells is the duration of the action potentials, which last about 300 ms, the same duration as the contraction. Consequently, contraction summation becomes then impossible even at high frequencies. This property is essential to prevent anarchic rhythm of heart contractions. Although the contraction of the heart muscle is myogenic, vegetative innervation and certain hormonal factors influence the frequency and force of heart contraction. [6]

The heart-brain axis is a complex system that warrants further examination. The relationship between the heart and the brain has been known for centuries, as evidenced by examples of death caused by extreme emotions [7]. Some studies have demonstrated the involvement of numerous brain areas in the modulation of cardiac functions, including the cingulate cortex, amygdala, parabrachial nucleus, hypothalamus, periaqueductal grey matter, the anterior insula, and some areas of the spinal cord (rostral ventrolateral medulla, RVLM). These areas are implicated in cardiac activity through sympathetic and parasympathetic systems through the formation of synapses with cardiomyocytes (Figure 8) [8].

From a clinical perspective, the heart-brain axis can be delineated as follows:

- The impact of cardiovascular disease on the nervous system
- The impact of neurological disorders on the cardiovascular system [7]

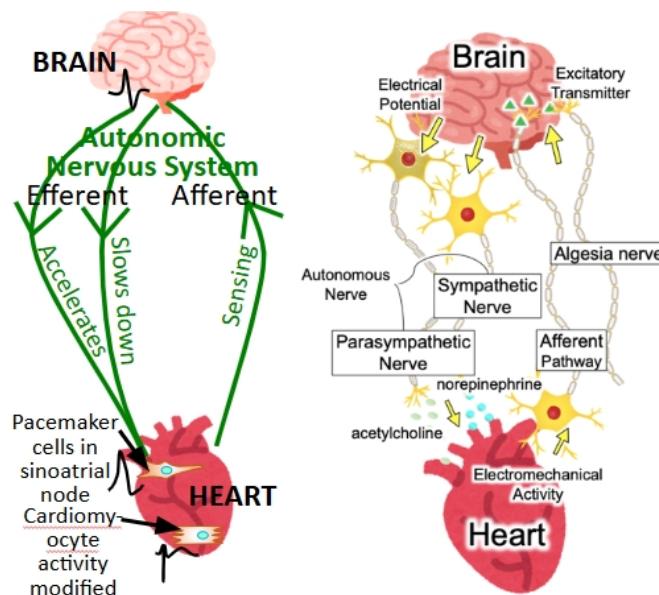


Figure 8: Neural control of the cardiovascular system - Contrôle neuronal du système cardiovasculaire [e]

II.1.c. The neuro-cardiac synapse is still poorly understood

The neurological consequences of cardiac disorders have been known for a considerable period of time; however, research into the pathophysiology underlying the brain-heart axis has only started in recent decades [7]. The neuro–cardiac junction (NCJ), which is the major structure of this system, remains poorly understood, with only a few cell models having been studied despite its significant role in cardiac activity modulation [9].

The paucity of studies about the NCJ is likely attributable to technological constraints, as microfluidic and electrophysiology techniques are only recently available. Furthermore, the regulation of cardiac activity through neurons is intricate and non-linear, effects can vary with numerous factors (environmental, psychological, ...).

From what we know, norepinephrine (NE) is released in the synaptic cleft in a diffuse manner following an action potential into the presynaptic terminal. Voltage-gated calcium channels are stimulated, thereby facilitating the influx of extracellular calcium in the neuronal cell. The calcium influx is responsible for the exocytosis of NE within the synaptic cleft. Following its release, NE binds with three types of receptors: alpha-1, alpha-2, and beta. The remaining NE is then rapidly recaptured by the **NE transporter**² or can be degraded intracellularly or in the synaptic cleft by the enzymes monoamine oxidase (MAO) or catechol-O-methyltransferase (COMT) (Figure 9) [22]. This mechanism is essential to limit the duration and intensity of NE on the cardiac cells. Improper evacuation of NE can lead to cardiac insufficiency and tachycardia [15]. Within the synapse, the release of neurotransmitters is subject to regulation by feedback loops that modulate secretion according to necessity. [15] The predominant mechanism of NE action on cardiomyocytes is associated with the stimulation of β 1-adrenergic receptors (AR) which activates the Gs protein. This protein elevates the enzymatic activity of adenylate cyclase, which converts ATP into cAMP. As a result, the cAMP level increases in the cytoplasmic compartment closely abutting the plasma membrane, where the stimulated β 1-AR are located. Simultaneous activation of multiple receptors distributed over the entire membrane surface leads to a global increase in cAMP in the cell.

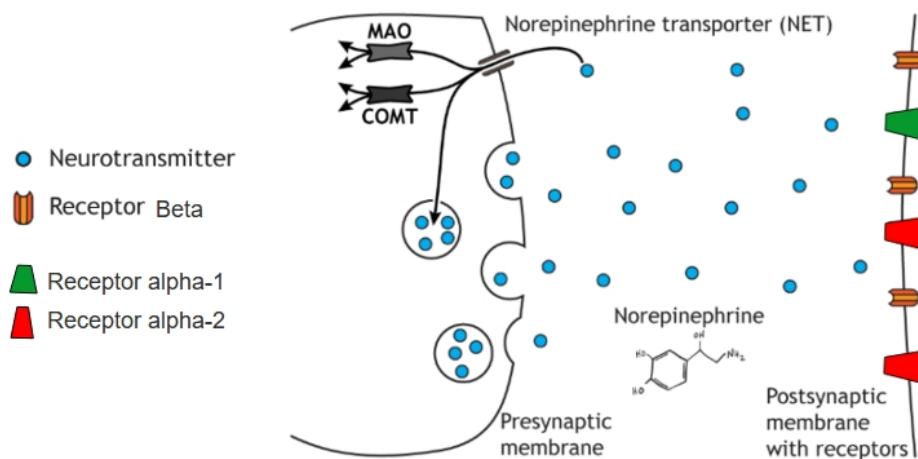


Figure 9: Norepinephrine action and reuptake in a synaptic cleft, modified from [h] - Action de la norepinephrine et élimination dans une fente synaptique, modifié de [h]

Other neurotransmitters are involved in sympathetic cardiac regulation, such as histamin and betanicotinamide adenine dinucleotide (β NAD), which could play a role in cardiac function regulation. It has also been proven that the neuropeptide Y (NPY) can have long-term effects such

² This transporter, also called NET, is a sodium-chloride-dependent transporter that mediates the reuptake of norepinephrine into the presynaptic terminal, where it can either undergo degradation or storage in vesicles. [22]

as promoting cardiac cells hypertrophy due to the prolonged Y5 receptor stimulation from chronic hyperactivation of the sympathetic system [15].

With electron microscopy, it has been shown that the synaptic cleft of NCJ is narrow, measuring less than 100 nm. The NE liberation concentration is estimated to be about 100 nM in the synaptic cleft. NCJs formation also depends on a lot of specific adhesion molecules to permit a better anchorage (Figure 9). [15]

II.1.d. Use of new method for in vitro studies : microfluidic and electrophysiology

In vitro studies are constrained by technical challenges, primarily due to the absence of standardized *in vitro* model. Consequently, approaches are specific to a laboratory or a study. *In vitro* platforms are often simplified compared to actual biological processes, yet they offer distinct advantages such as proximity with the cells and a more controlled environment along with economical and ethical benefits.

Microfluidics is one of the most promising technologies for the creation of *in vitro* synapses, offering numerous advantages over conventional methods, including more precision in chemical and physical environment controls, as the spatio-temporal neurotransmitter release in synapses is intractable to replicate with classic laboratory equipment. However, with microfluidic devices, it is now easier to generate neurotransmitter gradients closer to reality. It also allows co-culture of different cell types in dedicated microchannels, to study their interaction in conditions closer to *in vivo* (Figure 10). With proper imaging equipment it also makes it possible to track in real time cells responses. Microfluidics is a technology easily integrated with other types of technologies, such as biosensors, advanced microscopy, and electrodes, allowing a more in-depth study of cells. This type of co-culture (cardiomyocytes-neurones) is the next step for the laboratory [11][10].

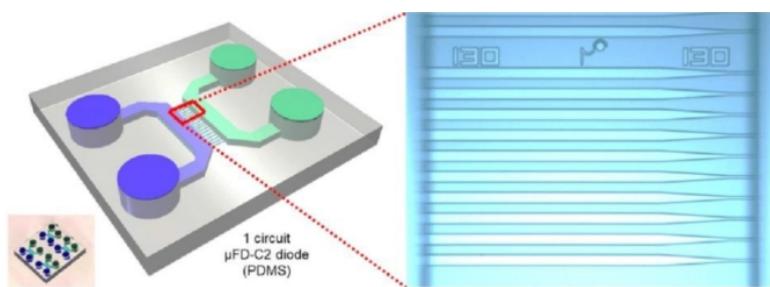


Figure 10: Example of microfluidic device for co-culture (OOC) - Exemple de dispositif microfluidique pour la co-culture (Organe sur puce) [9]

Electrophysiological recordings are also a fundamental step in specific cell studies such as cardiomyocytes. Most of the time, electrophysiology measurements are made with commercial multi-electrodes arrays (MEA) (Figure 11). Electrophysiology is used to record action potentials of groups of cells in real time. MEA are therefore useful for a non-invasive approach for measurement of cell activity. By integrating this type of model into *in vitro* studies, harvesting cell activity data is more accessible. [12].

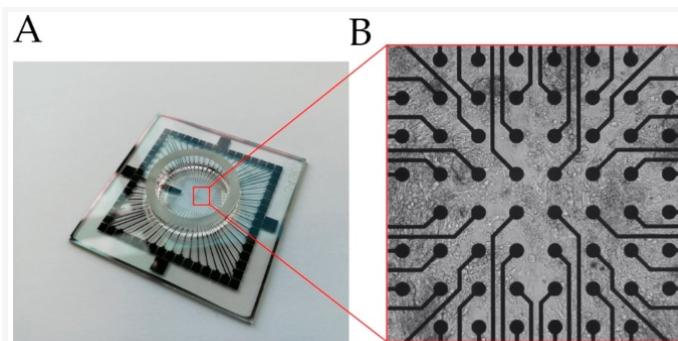


Figure 11: (A) Glass multi-/micro-electrode array (MEA) chip (B) Cells seeded on an MEA surface, grown on top of the electrodes (black dots) – (A) Puce MEA en verre (B) Cellules cultivées sur les électrodes (points noirs) d'un MEA [c]

II.2. Motivated problem

II.2.a. A growing proportion of heart diseases

“Deaths from cardiovascular disease (CVD) jumped globally from 12.1 million in 1990 to 20.5 million in 2021, according to a new report from the World Heart Federation (WHF). CVD was the leading cause of death worldwide in 2021, with four in five CVD deaths occurring in low- and middle-income countries”

Given the exponential growth of heart disease related deaths, there is an urgent need to enhance knowledge about the heart’s interactions with its environment, especially the neuro-cardiac interactions. “Over the past years, there is increasing evidence about the brain-heart interaction with major potential implications for treatment of cardiovascular diseases.” [13] . Cerebrovascular dysfunctions have been demonstrated to result in major cardiac rhythm disturbances. Emotional distress may provoke tachycardia, and “growing awareness of neuron cognitive decline in chronic vascular and congenital heart disease patients must challenge both clinicians and investigators”

II.2.b. Limits of MEA

As exposed previously, devices such as MEA are highly promising for research advancement on the brain-heart axis. However, although they are permitting extensive *in vitro* studies, they present limits still to be raised. The primary issue with MEA pertains to the dimension of the array, which is measured in μm and thus restricts its application to small mono-cell cultures due to the constrained surface area available for data acquisition. As the electrode density is low, spatial resolution is limited. Using a TFT platform offers a number of advantages that will be developed further, including enhanced spatial resolution (Table 1).

Table 1: Advantages and inconvenient of MEA and TFT array technologies - Avantages et inconvenients des MEA et TFT

	MEA	TFT array
Variety of sensors/measurement	++	+++
Spatial resolution	+	+++
Size of the culture	+	+++
Sensibility	+++	+
Price	++	+++
Interoperability	+++	+

Both MEA and TFT devices measure extracellular signals since the electrodes are not directly in the cells. The cells are grown directly on the electrodes, and the signal measured is therefore the extracellular signal generated by several simultaneous action potentials produced by the cardiomyocytes. In this study, only the extracellular signal will be recorded (Figure 12).

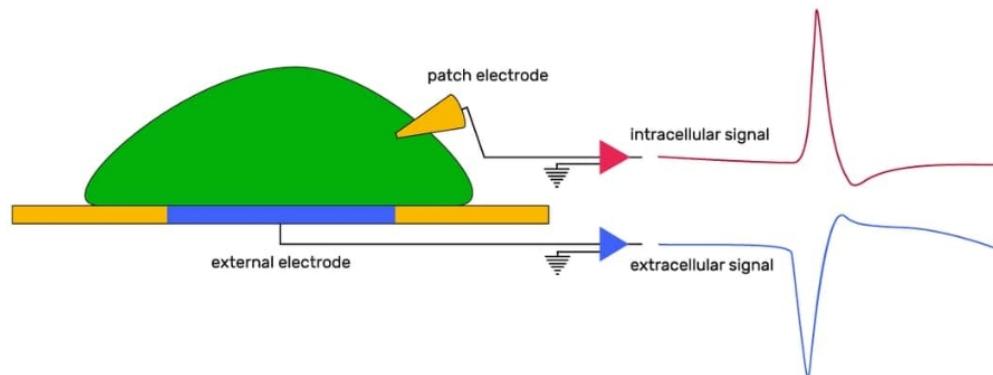


Figure 12: Different type of electrodes encountered in electrophysiology recordings [i]- Différents types d'électrodes rencontrées lors de mesures électrophysiologiques [i]

II.1.c. Numerous *in vivo* studies, few *in vitro* studies

The vast majority of studies on the heart-brain axis and the NCJ have been performed *in vivo* due to the fact that this type of system is difficult to recreate faithfully *in vitro*, since the dynamical environment of the heart is complex and a large number of parameters are involved.

In vivo studies remain expensive and questionable in terms of ethics, due to the high rate of animals used for a single study, whereas *in vitro* studies allow cell culture and growing and use in general less amount of animals. Besides economical and ethical considerations, *in vivo* results can be influenced by uncontrollable external factors including health and condition of the subjects, and the human-animal reproducibility is not always accurate due to interspecies variation in models. [10]

II.3. Targeted objectives

II.3.a. Protocol development and experimentation

The first objective of this internship is to explore the interaction between cardiomyocytes and neurotransmitters using an *in vitro* sensing platform. Therefore, one of the first steps is to become familiar with the platform and the cell culture techniques used in the lab. Once that was done, the next step consisted of targeting molecules of interest using previous studies. With the molecule of interest identified, it is then necessary to construct an experimental protocol focusing on different aspects such as the repeatability, the expected results, the costs, the material needed, and the difficulties to be encountered. Therefore, I planned on conducting my experiments on NE interaction with cardiomyocytes with different experimental conditions using the Bio-TFT sensor platform developed by the lab to attempt to demonstrate any direct effect of noradrenaline on cardiomyocytes *in vitro*.

II.3.b. Prototyping and testing a semi-microfluidic platform for experiments

Since such experiments with neurotransmitters have never been conducted in the Tixier-Mita Lab, one of my objectives was to create with the existing material a new semi-microfluidic device to carry out my experiments while ensuring compatibility with devices already developed in the laboratory (in particular, Bio-TFTs). Refer to **Annex I: Ideation, prototyping and final microfluidic device used for experiments** for further information on this aspect of my internship.

II.3.c. Data gathering and analysis

I plan to carry out an optical, electrical, and chemical study; therefore, I will use some software created by a former student for the optical analysis, as well as python code developed for my specific experiments. The electrical and optical data gathered will then be analysed and verified using comparative analysis. The results and data collected will also serve as a basis for comparison for the laboratory's next stage: co-culture of cardiomyocytes and neurons on the TFT devices developed.

III. Materials and methods

III.1. Biological Material

III.1.a. Source of cardiomyocytes

The cardiomyocytes cells used are **primary cardiomyocytes isolated from neonatal mouse** or their easy and quick harvest process and robustness. Since the lab does not have the authority to collect cells itself from living animals, the preparation of the cardiomyocytes was performed by collaborators Prof. Katsuhito Fujiu and Prof. Junichi Sugita, from the department of Cardiology at the University of Tokyo hospital.

III.1.b. Cell culture conditions

Cardiomyocytes are known to be anchorage dependent, which means that they attach readily to various types of surfaces for two-dimensional culture, such as ITO-covered glass or commercial T-25 flasks widely used for cell culture. The cells are cultivated in a medium composed of DMEM (Gibco), FBS, pen/strep and L-glutamine, thereby facilitating their growth and survival *in vivo*. The utilisation of these primary cell cultures enables the generation of new study material without compromising the well-being of the mice. They are placed in an incubator that maintains the temperature at 37°C and O₂ at 95%. It is essential to maintain aseptic conditions to limit contamination. All manipulations must be performed under a ventilated hood, wearing latex gloves, and all items brought under the ventilated hood must be cleaned with 70% ethanol. The medium needs to be changed every 1 to 2 days to ensure proper cell growth. When changing the medium, it must be reheated to 37°C.

III.2. Experimental device and technologies involved

III.2.a. TFT Device

The sensing platform used in the Tixier-Mita Lab is an *in vitro* array for electrophysiological and electrochemical investigation employing TFT technology on an ITO-covered glass substrate. A key benefit of TFT technology is its capacity to achieve a high density of transparent microelectrodes on a larger surface than MEA thereby facilitating more precise measurements, such as single-cell measurements (Figure 13) [23].

The surface area of a single electrode is 100 µm x 100 µm. Electrodes are made of ITO, a material that confers both transparency and electrical conductivity. The electrodes are arranged on glass. Each electrode is connected to a TFT used as an on/off switch. As is typical of most transistors, TFT transistors consist of a drain, a gate, and a source. Each TFT's drain is connected by rows and controlled by a **digitiser**, combined with an amplifier. The connection between TFT and digitiser is made by a **multiplexer**. Each gate is connected by column to an output 12V DC voltage provided by the **JAPASTIM**, and the sources are connected to each individual electrode. The TFT substrate

is wire bonded to a PCB that has been previously grounded to the dimensions of the substrate. The PDMS cells chamber is fixed with additional PDMS to stabilise both the chamber and the wire connections (Figure 13) [23]. This technology is at the heart of a powerful multimodal *in vitro* measurement platform.

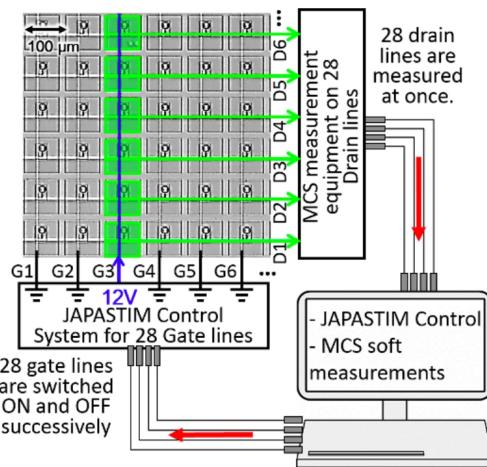
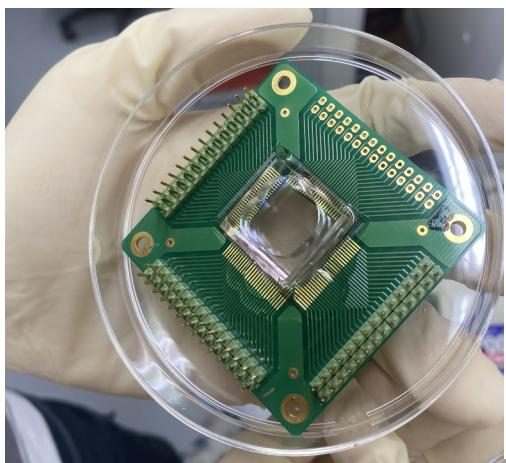


Figure 13: TFT Device with PCB and PDMS cell chamber (Left), TFT device operating diagram (Right) - Dispositif TFT avec PCB et chambre cellulaire en PDMS (Gauche), Schéma du fonctionnement du dispositif TFT (Droite) [23]

III.2.b. Microfluidics

Despite the fact that the Tixier-Mita Lab main activity is not focused on microfluidics, the use of such technology can be advantageous in certain experiments, such as a precise and controlled flow rate when introducing chemicals into the cell chamber. A specific semi-microfluidic device (Figure 14) has been designed for experimentation with neurotransmitters. This apparatus includes a syringe pump (KDScientific Legato 210) for administration of chemicals/medium and a TP-5SA AS ONE micro-pump to expedite medium drainage. This versatile setup can be placed in an incubator or under a microscope without compromising its functionalities.

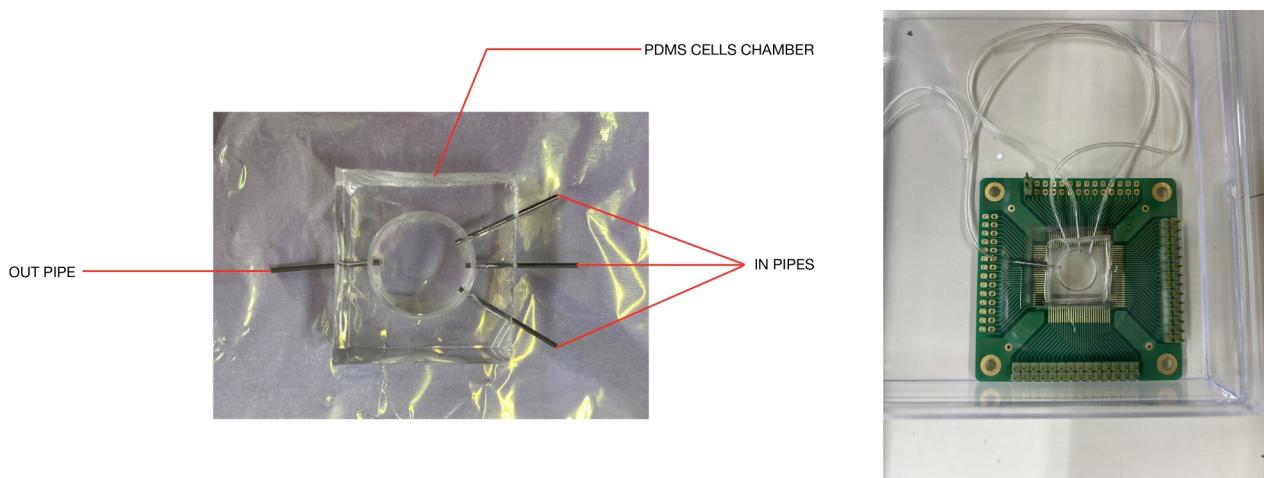


Figure 14: Prototype of the PDMS chamber used for experiments - Prototype de la chambre en PDMS utilisée pour les expérimentations

The optimal flux rate was determined by means of a Python program that calculated the number of blue pixels per frame for a volume of 41 μL (see III.4.a for the calculation of this volume).

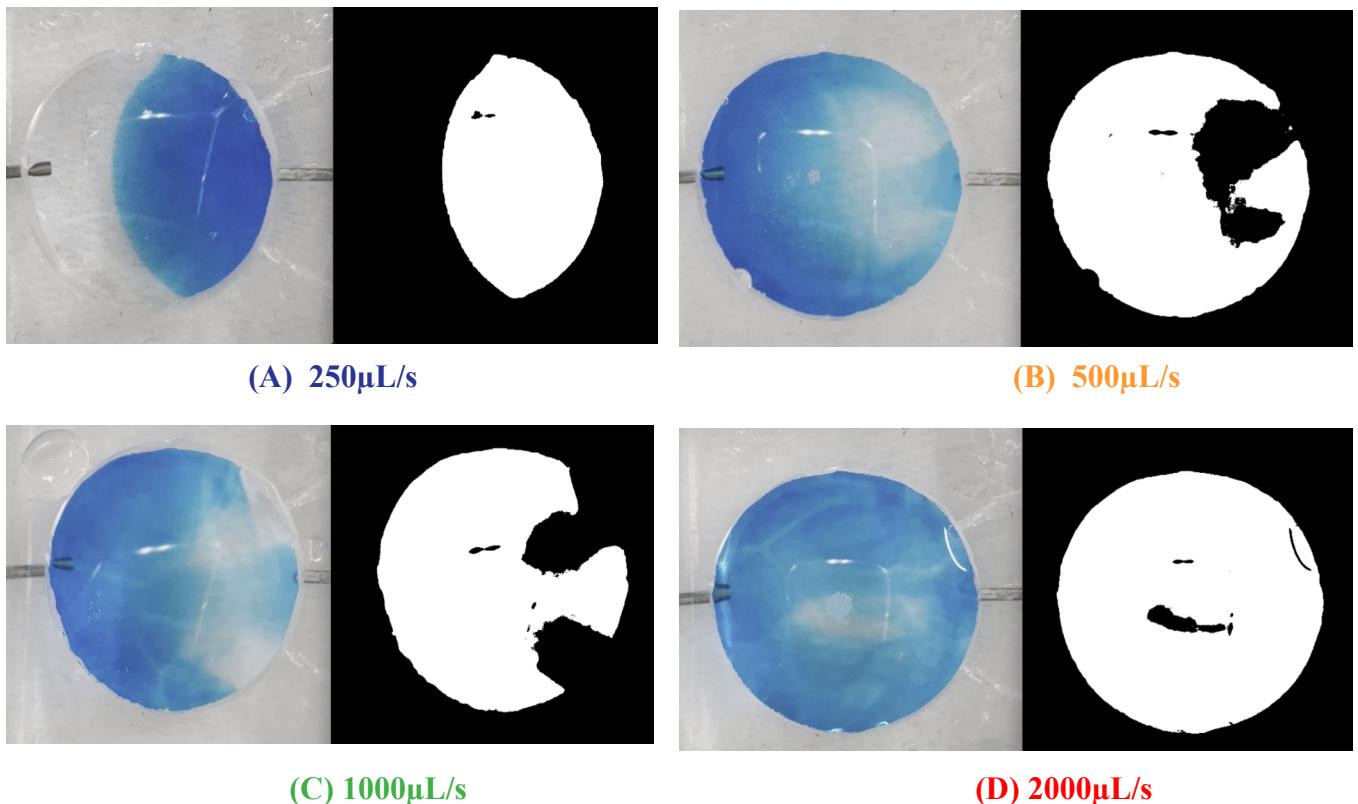


Figure 15: Flux at 23 second, 41 μL in, original frame (right) and masked HSV frame (left) for speed of $250\mu\text{L}/\text{min}$ (A), $500\mu\text{L}/\text{min}$ (B), $1000\mu\text{L}/\text{min}$ (C) and $2000\mu\text{L}/\text{min}$ (D) – Flux à 23 secondes, 41 μL frame originale (droite) et frame HSV avec masque (gauche) pour des vitesses de $250\mu\text{L}/\text{min}$ (A), $500\mu\text{L}/\text{min}$ (B), $1000\mu\text{L}/\text{min}$ (C) et $2000\mu\text{L}/\text{min}$ (D)

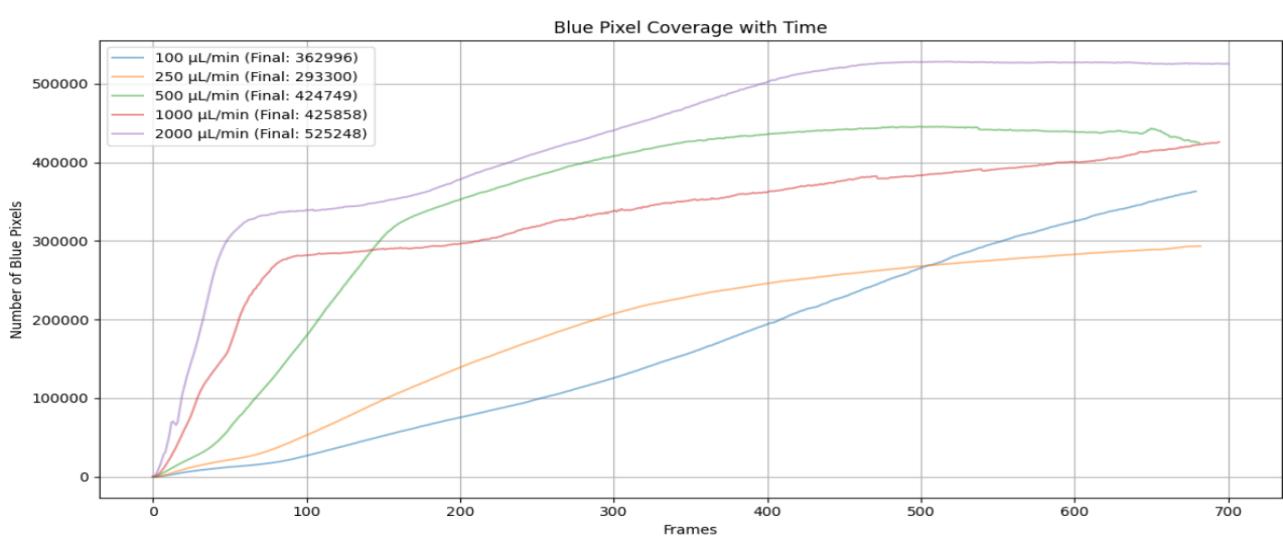


Figure 16: Evolution of blue coverage at 150 $\mu\text{L}/\text{min}$, 250 $\mu\text{L}/\text{min}$, 500 $\mu\text{L}/\text{min}$, 1000 $\mu\text{L}/\text{min}$, 2000 $\mu\text{L}/\text{min}$ with time - Evolution de la couverture du liquide bleu selon le temps pour des vitesses de 150 $\mu\text{L}/\text{min}$, 250 $\mu\text{L}/\text{min}$, 500 $\mu\text{L}/\text{min}$, 1000 $\mu\text{L}/\text{min}$, 2000 $\mu\text{L}/\text{min}$

The syringe pump KDS 210 Legacy was utilised to diffuse neurotransmitters through the pipes. The wasted volume, calculated using the length and radius of the pipe (around 210 µL), was added to the volume supposed to be released if the pipe was empty or filled with something other than neurotransmitters. A speed of **2000 µL/min** was selected for the experiments because it yielded the optimal final coverage in blue pixels while considerably reducing the exposure time to norepinephrine, making it easier to adjust this parameter (Figure 15 & 16).

III.3. Cell culture protocols

Following the receipt of the cardiomyocyte cells, they are initially counted using a BioRad TC20 automated cell counter before being subjected to centrifugation at 300 x g for 5 min using a Thermo Scientific™ Sorvall™ ST8 FR centrifuge. Thereafter, the cells are diluted with medium to achieve the desired concentration of 8.10^6 cells/mL. Once the desired concentration is reached, the cells are ready to be seeded. They are directly poured in the cells chamber with a micropipette (approx. 1mL) and stored in the incubator. It is imperative to replace the medium at regular intervals of 1-2 days. The remaining cells are stored in T-25 flasks and the medium is changed if required. All manipulations involving the opening of the petri dish lid must be carried out under a ventilated sterile hood. For a more detailed protocol on cell seeding and storage, refer to **Annex III: Cells seeding, storage and maintenance**.

Prior to and following the use of the TFT devices, they are meticulously cleaned by being immersed in ethanol for a period of 10 minutes and subsequently rinsed with ultra-pure water. The PDMS cells chamber is fabricated by blending the polymer with its polymeriser in a 1:10 proportion. The mixture is then placed in a vacuum chamber to break all bubbles before being poured onto a dish plate and polymerised for 30 minutes at 90°C. Following this, the PDMS is then cut to size using a cutter, and the cell chamber is punched using a 100 mm biopsy needle. Microfluidic channels are then punched with a 0.5 mm biopsy needle, after which metal pipes are inserted into the resulting apertures. The chamber is then bonded with the glass (or TFT device) by plasma bonding.

III.4. New experimental methods protocol

III.4.a. Choice of solvent and shelf stability of the neurotransmitter solution

In order to store the norepinephrine (L-Noradrenaline Bitartrate Monohydrate A0906, TCI), it is necessary to dilute it in a solvent and freeze it at -80°C, protecting it from light, since norepinephrine is not very stable in solution. With regard to the solvent, two options were available: organic and aqueous. The aqueous solution was determined to be the optimal choice in terms of cell viability; however, there is a paucity of studies investigating the long-term stability of these solutions, with the majority of research conducted over 24 to 48 hours [24]. Conversely, the organic solvent was identified as the most suitable option in terms of shelf-life duration, exhibiting 6-month stability up to 30 mg/mL (solubility of NE bitartrate in DMSO) [25], and was thus selected for further investigation. In order to ensure that the solvent would not harm the cell culture, the stock solution was highly concentrated, then diluted several times to permit large dilution of the solvent in the medium. Once the stock solution was ready, it was stored in several small aliquots for defrosting when needed to avoid wastage, then diluted in PBS. Precaution must be taken while handling norepinephrine in all its forms since this substance is highly hazardous (refer to **Annex II: Risks, precautions and individual protection measures**). It is imperative to work in a well-ventilated area and to wear protective equipment, including glasses, gloves, masks, and a lab coat.

The stock solution concentration was determined as follows:

Given the solubility of NE bitartrate in DMSO (approx. 30 mg/mL), the maximum molarity of the solution in norepinephrine bitartrate (molar mass : 337.28 g/mol) can be calculated:

$$C_{max} = \frac{30}{337.28} = 88.95\text{ mM}$$

In order to avoid any saturation of the solution and to optimise the stock solution stability, a mass concentration between 20 and 25 mg/mL will be selected and the volume needed of DMSO for 250 g of norepinephrine bitartrate will be determined:

$$V_{DMSO} = \frac{250}{21} = 11.9\text{ mL}$$

The range of solutions was prepared as follows:

The bitartrate was weighed on a precision scale (0.250 g) in a fume hood. 11.9 mL of DMSO was measured and mixed with the bitartrate until dissolution, with the necessary safety precautions being observed. The solution was then stored in aliquots of 1 mL at -80°C (12 x 1 mL aliquots), with the aliquots being protected from UV light. The solution was found to be 62.23 mM. In order to utilise the stock solution, it is necessary to carry out further dilution. Firstly, a preliminary aqueous solution can be prepared by diluting the stock solution to a concentration of 1 mM (16 µL of the initial solution, 984 µL of PBS for 1 mL). The second intermediate solution should have a concentration of approximately 5 µM in norepinephrine bitartrate (dilution 200:1).

Concentration in pure norepinephrine can be calculated :

$$C_{NE} = \frac{5 \times 169.18}{337.28} = 2,508 \mu M$$

The cell chamber is filled with 1mL of medium, and the desired concentration of norepinephrine is 100 nM. The requisite amount of intermediate solution needed can be easily determined:

$$V_{NE} = \frac{(C_f \times V_{ch})}{(C_{NE} - C_f)}$$

$$V_{NE} = \frac{(0,1 \mu M \times 1000 \mu L)}{(2,508 \mu M - 0,1 \mu M)} = 41,5282 \mu L$$

With, as calculated before, 209,6320 μL of wasted solution (pipe volume), the volume entered on the syringe pump must be $41,5282 + 209,6320 = 251,1602 \mu L$. To ensure the accuracy of the dosage of norepinephrine, the solution is coloured blue to provide visual traceability, since the placement of the syringe on the pump can introduce measurement errors such as air bubbles.

III.4.b. Choice of the best chamber drainage protocol

Given the brief release time of norepinephrine in the synaptic cleft is very short, as it is immediately recaptured by norepinephrine transporter [15] , one of the focal points of this study was to limit the time of action of norepinephrine *in vitro*. Given the complexity of transporters' use *in vitro*, a mechanical wash of the cell chamber was performed by emptying the medium containing NE with a TP-5SA ASONE pump (Figure 17), rinsing the chamber with PBS, and it was filled with fresh medium using pre-filled syringes directly connected to it.



Figure 17: Harrick benchtop plasma cleaner (Left), ASONE Micropump (Right) - Nettoyeur plasma de table Harrick (Gauche), Micro-pompe ASONE (Droite)

The cells' chambers were bonded with a glass plate (which had been washed with acetone and subjected to ultrasounds) using a Harrick benchtop plasma cleaner (Figure 17). Cardiomyocytes were cultivated on the device for a period of seven days before the introduction of norepinephrine . Several rinsing protocol were considered prior the addition of the fluorescent probe (Table 2).

Table 2: Protocol used for NE elimination - Protocoles utilisés pour l'élimination de la NE

- Rinsing with medium	
<p>Syringe pump</p>	Step 1: Introducing NE for 10 seconds Step 2: Draining the cell chamber Step 3: Filling the chamber with heated medium Step 4: Draining the chamber (repeat 3x step 3-4) Step 5: Fill the chamber with heated medium
- Rinsing with PBS	
<p>Syringe pump</p>	Step 1: Introducing NE for 10 seconds Step 2: Draining the cell chamber Step 3: Filling the chamber with heated PBS Step 4: Draining the chamber (repeat 3x step 3-4) Step 5: Fill the chamber with heated medium
- Natural oxidation (UV + O ₂)	
<p>Syringe pump</p>	Step 1: Introducing NE for 10 seconds Step 2: Expose the chamber to light and O ₂
- No rinse	
<p>Syringe pump</p>	Step 1: Introducing NE

An ELISA test was then carried out to interpret the experimental results, employing the Competitive Norepinephrine ELISA Kit (ab287789), Abcam. The results were then analysed by an AsOne plate reader which had been rented for the specific purpose of conducting this experiment. The configuration of the plate is illustrated in Figure 18.

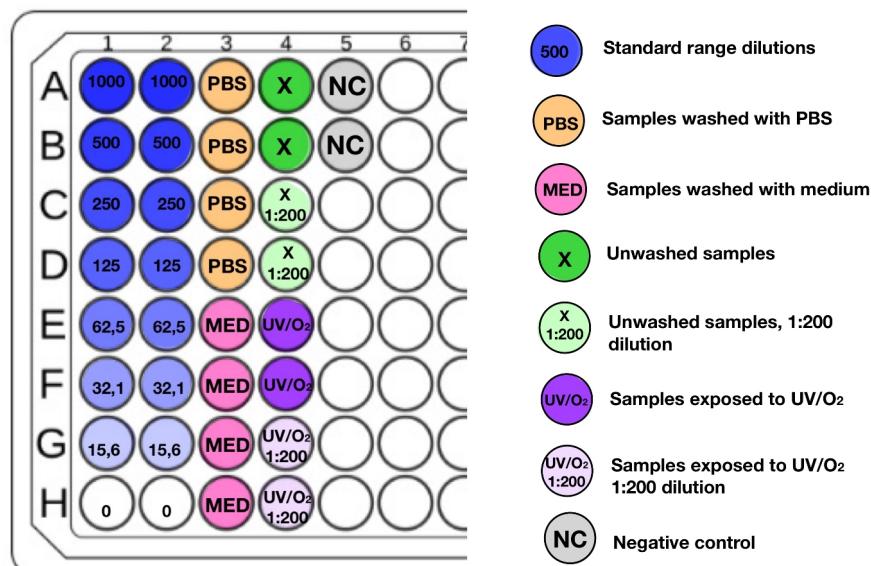


Figure 18: ELISA test 96 well plate configuration - Configuration de la plaque 96 puits du test ELISA

In order to ensure the fidelity of the assay, each sample was tested in a minimum of two wells. It was hypothesised that the unwashed samples and the samples exposed to UV and O₂ would be off range. To facilitate the reading of results, further dilution at the rate of 1:200 was performed.

As anticipated, the unwashed samples and the samples exposed to UV/O₂ were found to be off-range (i.e. with an absorption of 1). However, the diluted samples exhibited an approximate concentration between 450 and 5500 pM (i.e. between 90 and 110 nM), which is equivalent to the initial concentration. Consequently, these protocols were deemed inconclusive and will not be utilised further. It was observed that PBS and medium washing protocols yielded analogous outcomes, exhibiting minimal absorption. The measured concentration in both cases was less than 15 pM, which can be considered negligible in comparison with the base concentration of 100 nM.

Given the observation that PBS and medium wash experiments yielded analogous results, the protocol employing PBS was selected. This decision was made with the objective of minimising the waste of medium, a factor that is more economical in terms of cost and more straightforward in terms of preparation when using PBS.

III.4.c. Main experiment

Subsequent to the conclusion of the preliminary experiments, the initiation of the main experiments was possible, which comprised the optical and electrical measurement of cell behaviour in the presence of norepinephrine. The following diagram (Figure 19) illustrates the overall protocol for the experiment, reproduced with different variable configurations (Table 3).

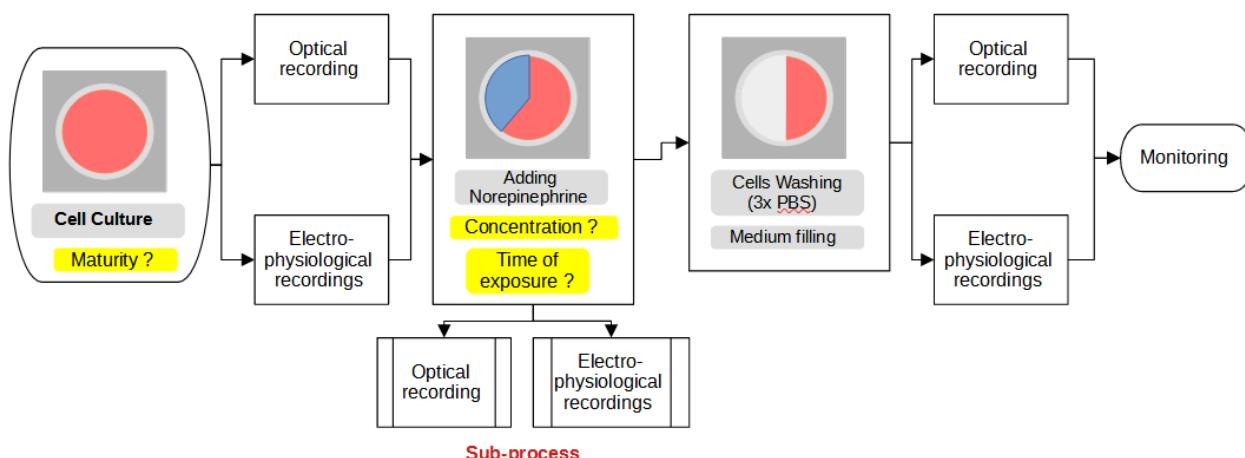


Figure 19: Overall scheme of the main experiment course - Schema global du déroulement de l'expérience principale

Table 3: Parameters Tested³ - Paramètres testés

Concentration→	50 nM		100 nM		200 nM		500 nM	
Time of exposure ↓	3 days old	5 days old						
Immediate wash			X	X				
1 minute			X	X				

Following a four-day monitoring period, experiments were conducted on cell shape and viability. These experiments incorporated the utilisation of BioTracker 490 Green Cytoplasmic Membrane Dye fluorescent probe for the analysis of cell viability. Snapshots of the fluorescent observation were made with the same parameters (2x objectives, 100% light intensity, 8 bit colour scale 30 to 255, exposure 35.7 ms, gain 8x) and thus analysed with ImageJ. In addition, trypan blue cell counting was performed. In addition, a series of optical observations were conducted to ascertain the general shape and state of the cell, with the objective of detecting the slightest abnormality. The optical recording of the experiments was conducted using a Photometrics Cascade II microscope camera, with subsequent treatment conducted using ImageJ, Anytrack and Python. The

³ Due to lack of time, resources and contamination, all parameters could not be tested as expected. Experiments with 5 days old cultures will be conducted from January 28th, the results cannot be presented in the report by February 3rd

beating pattern of the cells can be optically characterised using Anytrack software (developed by a former trainee). A fixed point tracker was established as a static reference, and a template tracker was incorporated in regions where beating was discernible. Subsequent to the delineation of the detection zone, the software traced the temporal variation of the distance between the two trackers (in pixels) (Figure 20).

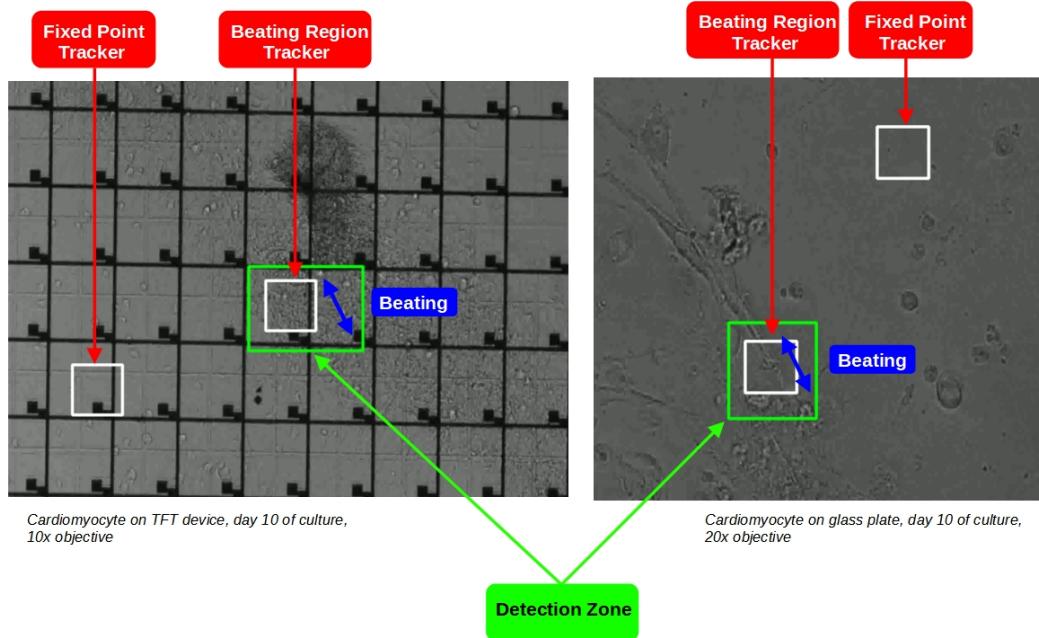


Figure 20: Positioning displacement trackers on videos using Anytrack software - Positionnement des traqueurs de déplacement sur des vidéos avec le logiciel Anytrack

Electrophysiological recordings were made in the same time using a bespoke setup (Figures 21 & 22). The setup involves the use of a control card called “JAPASTIM” (used for TFT gate control). Control of JAPASTIM PCB is made with Tera Term 5 software. Electrophysiological records are acquired using a digitiser (used for TFT drain control), thus displayed with McRack software and treated with Python and LabPlot2.

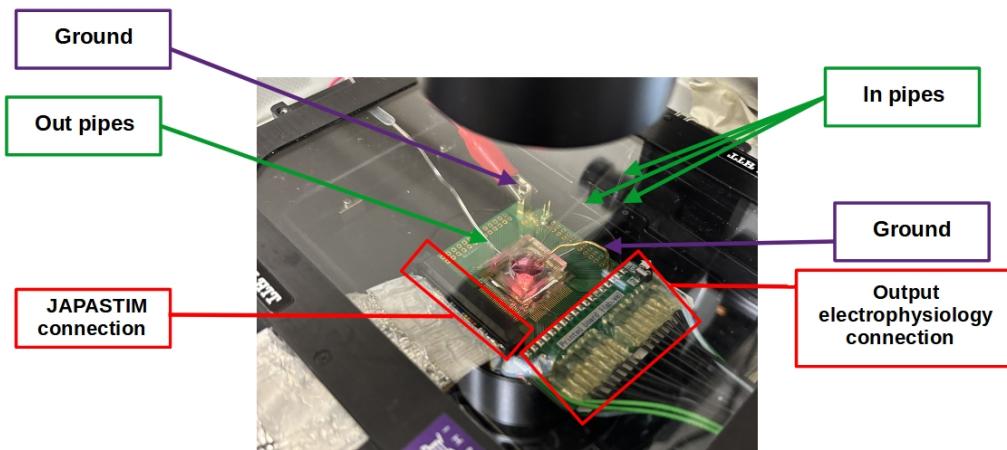


Figure 21: Experimental setup, zoomed on TFT device - Installation expérimentale, zoom sur le dispositif TFT

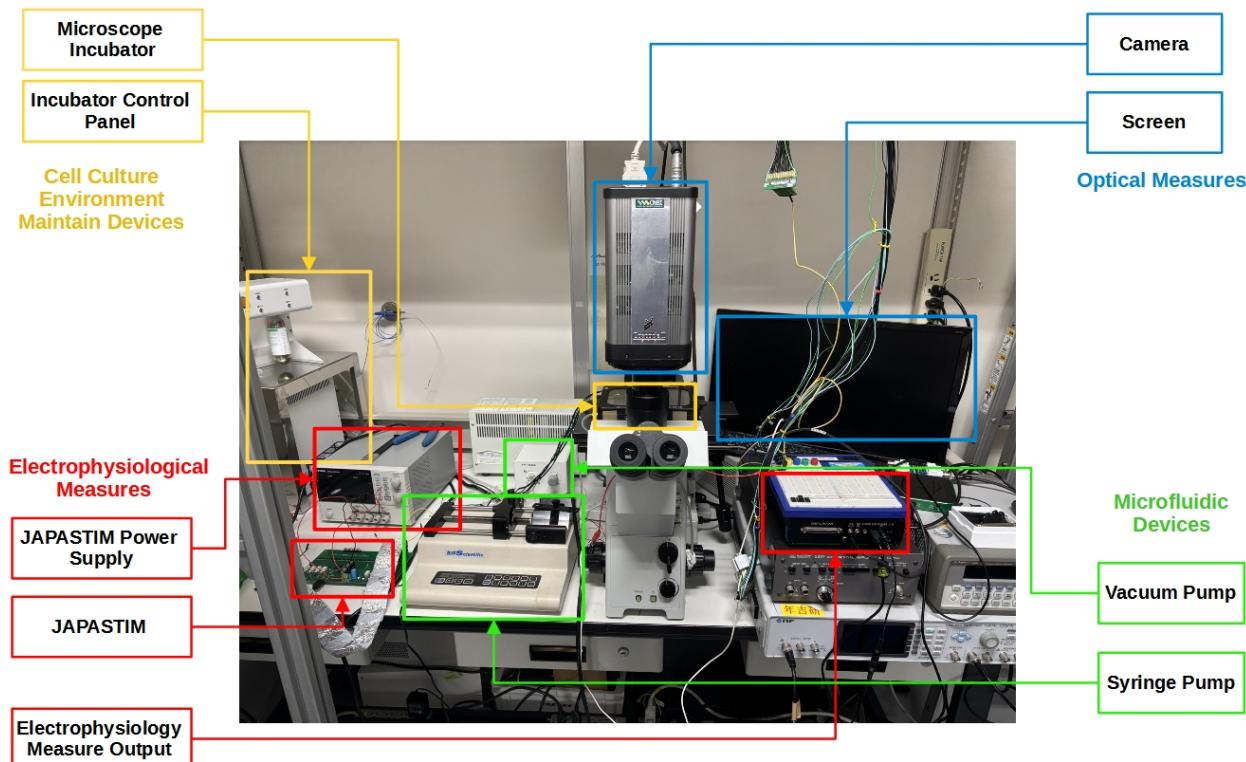


Figure 22: Experimental Setup - Installation expérimentale

III.5. Safety and precaution

III.5.a. Contamination

As cell culture conditions are highly conducive to contamination (high humidity, 37°C temperature...), many precautions must be taken during handling to avoid cross-contamination of the different cultures. Any manipulation involving opening the Petri dish lid must be performed under the ventilated hood; hands must be covered with gloves at any time and rubbed with 70% ethanol before and after any manipulation of the culture. A further cleaning of any sensitive work area, with ethanol and UV-light disinfection is mandatory after any experiments.

Furthermore, any device involved in cell culture has to be autoclaved (when possible) and cleaned with ethanol before using it for cultivating the cells. Any consumables must be sterile (T-25 Flasks, micropipette tips, tubes ...).

III.5.b. Bio-hazard

Norepinephrine is a highly hazardous chemical and must be handled with precautions to avoid any impact on the operator's health. This involve working in a well ventilated area, wearing proper personal protective equipments and reading the safety data sheet provided by the selling organisation. An extract of this safety data sheet can be consulted on **Annex II: Risks, precautions and individual protection measures**.

IV. Results and discussion

IV.1. Results of the main experiments

IV.1.a. Normal cell shape and behaviour

Optical analysis

A plethora of observations of cardiomyocyte cultures under basal conditions have led to the conclusion that there is no regular beat pattern. In fact, these cells are very sensitive to variations in the environment. The observed beat will vary depending on the temperature, the freshness of the medium, the time spent outside the incubator, the culture time of the cells, and the proportion of fibroblasts in the culture, amongst other factors. It is imperative to analyse the cell culture under these basal conditions before each experiment in order to compare the results with the basal behaviour of the same culture. The results before and after the experiments therefore represent a paired sample. Examples of the different beat patterns encountered are shown below. **It should be noted that the cells may also beat very weakly or not at all**, which poses problems when carrying out certain experiments in the laboratory. In instances where the experimental focus does not necessitate analysis of the beating characteristics but rather the activity of the cells (e.g., a stimulation test), the addition of a beta blocker, such as isoprenaline, can be employed to enhance the strength and frequency of the cell beats. However, when the objective of the experiment is direct analysis of the beating characteristics, this method cannot be applied, as it alters the inherent properties of the natural beat.

As demonstrated in Figure 23, the 9-day-old cell culture exhibits a semi-regular beating pattern, with 2-4 beats per trigger, as indicated by the original signal and. In contrast, the 8-day-old culture (see Figure 24) displays a regular 3×3 beating pattern, with a dominant frequency of 0.35 Hz, as evident in both the original signal and the Fourier decomposition.

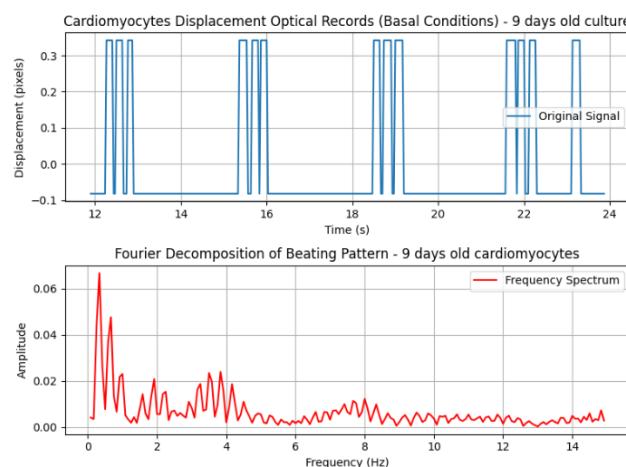


Figure 23: Beating pattern of an 8 days old cardiomyocyte culture A in basal conditions - Battements de cardiomyocytes A cultivés 8 jours en conditions basales

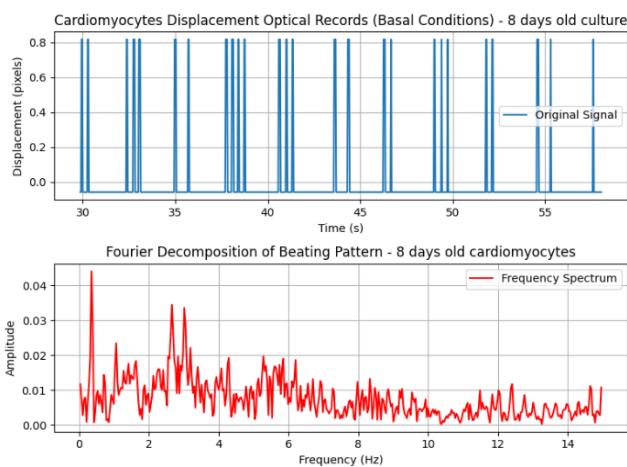


Figure 24: Beating pattern of an 9 days old cardiomyocyte culture B in basal conditions - Battements de cardiomyocytes B cultivés 9 jours en conditions basales

As demonstrated in Figure 25, this 3 day old culture displays a regular single-beat pattern with a predominant frequency of 0,87 Hz.

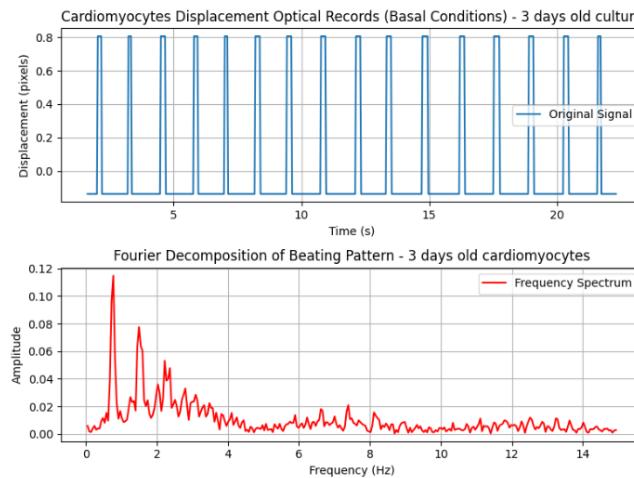


Figure 25: Beating pattern of an 3 days old cardiomyocyte culture C in basal conditions - Battements de cardiomyocytes C cultivés 3 jours en conditions basales

Electrophysiological analysis

The recording shown in Figure 26 is the external potential field signal⁴ signal of cardiomyocyte culture under basal conditions. Each step of the potential field can be identified on (Figure 26). Parasite negative spikes can be observed, and have been identified as JAPASTIM induced noise.

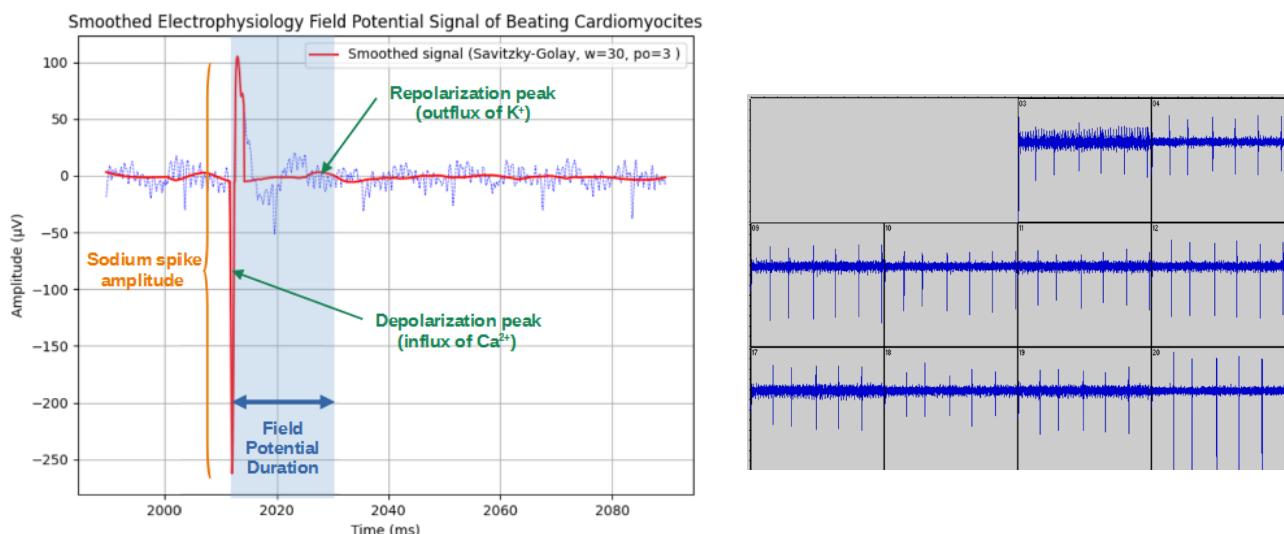


Figure 26: Electrophysiology recordings of cardiomyocytes potential field (filtered data), 7 day culture (Left) - Example of electrophysiology records of basal cell culture (McRack interface) (Right)
Mesures électrophysiologiques du potentiel de champs de cardiomyocytes (données filtrées d'une culture de 7 jours)(Gauche)- Exemple d'enregistrements électrophysiologique de culture en condition basale (interface McRack)(Droite)

⁴ Transitory electric signal generated by the synchronic cumulative electrical activity of single cells in a tissue. It can be referred to as the temporal derivative of the corresponding action potential. [18]

The rhythmic behaviour of cells can also be studied using electrophysiology via the TFT platform. The results of electrophysiological measurements carried out on cultures of healthy cardiomyocytes can be seen above (Figure 26). Each rectangle represents the real-time measurement of an electrode (McRack software interface). A total of 28x28 electrodes can theoretically be connected, but for practical reasons, around 10x10 are usually connected per device. Electrophysiology measurements offer a much higher temporal resolution, in the order of a millisecond, and on the whole, electrophysiology is more sensitive, making it possible to measure minimal variations in voltage directly, whereas optical measurements are based on observation of phenomena that are often mechanical. The frequency outcome regarding basal cell culture is supposed to be the same as with optical recordings.

IV.1.b. Cell behaviour during and after norepinephrine exposure

Table 4: Results of main experiment, 3 days old culture, 100 nM - Résultats de l'expérience principale, cultures de 3 jours, 100 nM

100 nM – 3 Days old cell culture				
Incubating Time →	Immediate wash		1 min rinse	
Optical analysis	Frequency-Amplitude		Frequency-Amplitude	
	Before:	4,08 Hz, 2px (regular periodicity)	Before:	Very few, isolated activity (few cells cluster beating at low frequency (0,3-0,5 Hz) most of the cells are inactive)
	5 min after:	1,80 Hz, 0,8 px (less regular periodicity)	5 min after:	No beating on observation zone
	After 3 days:	Few beating cluster observable (low frequency 0,3-0,5 Hz), turbid medium	After 4 days:	No Beating, turbid medium
Electrophysiology ⁵	Frequency-Amplitude- Signal Shape		Frequency-Amplitude- Signal Shape	
	Before:	3,97 Hz, 248,57 µV, Normal shape	Before:	No beating
	5 min after:	3,10-3,83 Hz, Irregularity (no beat for 10 s) 286,44 µV, Normal shape	Right after:	No beating
	After 3 days:	Noisy signal due to turbidity of the medium and cell death (low signal-to-noise ratio)	After 4 days:	Noisy signal due to turbidity of the medium and cell death (low signal-to-noise ratio)

⁵ Due to high noise introduction and movement during norepinephrine pouring and washing, electrophysiology and optical records during the experiments are hardly usable.

Results correlation	Similar frequencies observed in both electrophysiology and optical observations		No beating observed from each method	
General outcome 4 days after experiment	Fluo analysis:	0,5613 %	Fluo analysis:	0,3000%
	Cell counting:	8% live cells	Cell counting:	7-8% live cells
	Other observations:	Turbid and yellow medium, cells detachment	Other observations:	Turbid and yellow medium, cells detachment
Conclusion	Changes in regularity and frequency of cell activity after NE exposure. Death of the majority of the cell as a result of the experiment		Inconclusive results due to inactivity of the cells from beginning of experiment. Death of the majority of the cell as a result of the experiment.	

Table 5: Table 4: Results of main experiment, 5 days old culture, 100 nM - Résultats de l'expérience principale, cultures de 5 jours, 100 nM

100 nM – 5 days old				
Incubating Time →	Immediate rinse		1 min rinse	
Optical analysis	Frequency-Amplitude		Frequency-Amplitude	
	Measurements still ongoing		Measurements still ongoing	
Electrophysiology	Frequency-Amplitude- Signal Shape		Frequency-Amplitude- Signal Shape	
	Measurements still ongoing		Measurements still ongoing	
Results correlation	Measurements still ongoing		Measurements still ongoing	
General outcome 4 days after experiment	Fluo analysis:	Measurements still ongoing	Fluo analysis:	Measurements still ongoing
	Cell counting:	Measurements still ongoing	Cell counting	Measurements still ongoing
	Other observations:	Measurements still ongoing	Other observations:	Measurements still ongoing
Conclusion	Measurements still ongoing		Measurements still ongoing	

IV.1.c. Example of results graphs and data

Due to the substantial volume of data collected, not all the graphical results can be exposed. To illustrate, this section presents the results obtained from the experiment carried out with the following parameters: **3-day-old culture, 100 nM, immediate rinsing.**

Electrophysiological data

Curves are smoothed with a low-pass Butterworth filter (CF = 20-30 Hz, order = 4), combined with a spike detection function that preserves the integrity of the spikes (Python data processing).

Before adding norepinephrine

The electrophysiological signal (Figure 27) and the Fourier Transform (Figure 28) show that the cells are active and beat relatively regularly prior to the addition of norepinephrine in the medium. The dominant frequency (beating frequency) was found to be **3.97 Hz**. The total average amplitude of spikes was **248.57 µV** (average calculated over the entire measurement period). The shape of the peaks was standard, showing a positive and a negative peak, followed by a period of slow repolarisation before stabilisation at 0 (Figure 29).

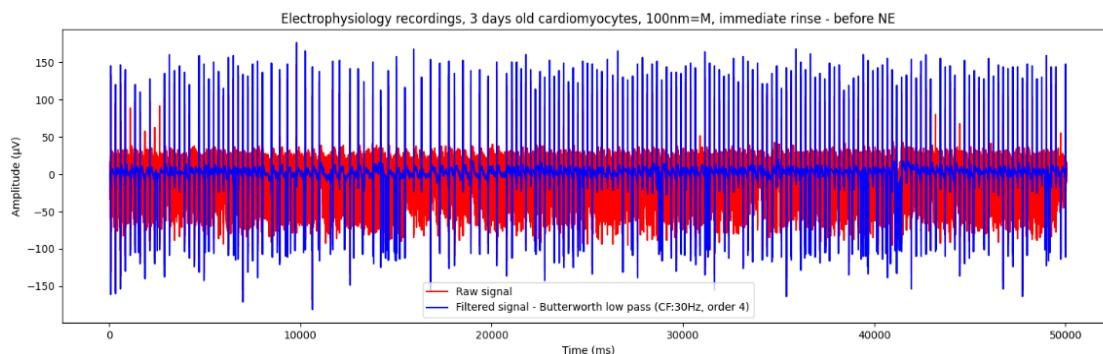


Figure 27: Raw (red) and filtered (blue) signal of cardiomyocytes field potential before norepinephrine – Signal brut (rouge) et filtré (bleu) du potentiel de champ des cardiomycites avant norepinephrine

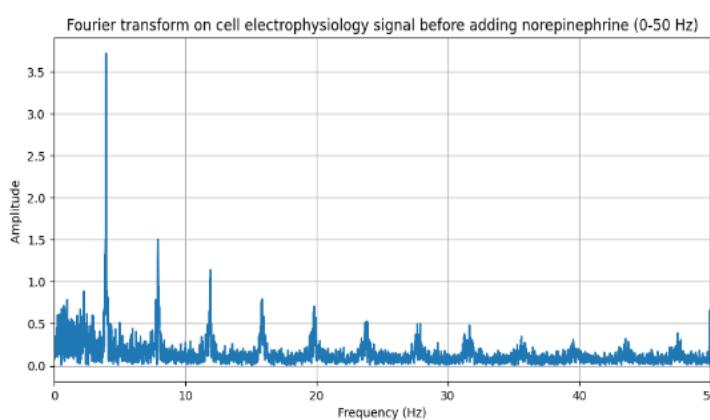


Figure 28: Zoom on Figure 28 signal - Zoom sur le signal de la Figure 28

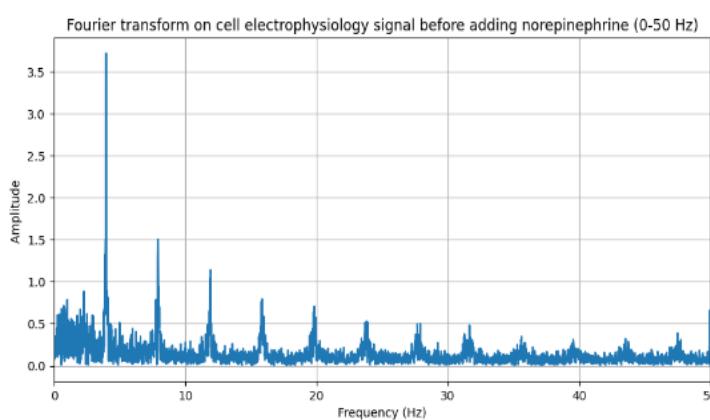


Figure 29: Fourier Transform of electrophysiology measurements before norepinephrine - Transformée de Fourier des mesures électrophysiologiques avant norepinephrine

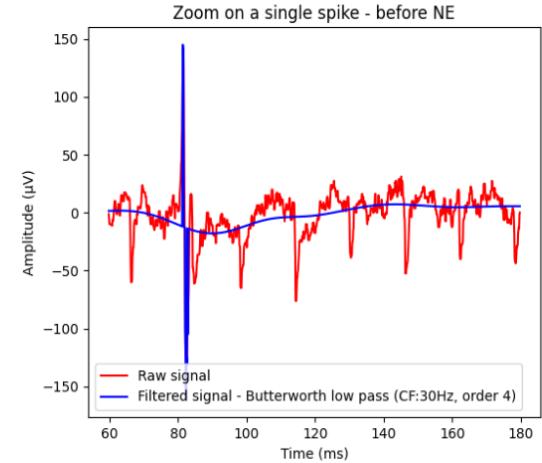


Figure 29: Fourier Transform of electrophysiology measurements before norepinephrine - Transformée de Fourier des mesures électrophysiologiques avant norepinephrine

10 minutes after adding norepinephrine (zoomed)

The electrophysiological signal (Figure 30), and the Fourier Transform (Figure 32 & 33) show that the cells are active. However, the beating pattern is not standard, showing a regular beating for the first 18 seconds (frequency = **3.83 Hz**), followed by a no-beating period between 18 and 30 seconds. Thereafter, the heart rate gradually resumes (frequency = **3.10 Hz**) and the signal becomes significantly more noisy, indicating a less regular beating pattern than before the administration of norepinephrine. The total average amplitude of spikes is 286.44 μ V (calculated over the entire measurement period). The shape of the peaks is standard, showing a positive and a negative peak, followed by a period of slow repolarisation before stabilisation at 0 (see Figure 31).

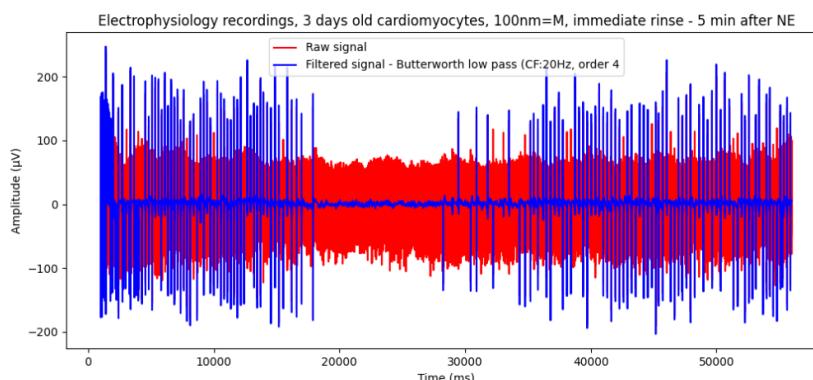


Figure 30: Raw (red) and filtered (blue) signal of cardiomyocytes field potential 5 min after norepinephrine – Signal brut (rouge) et filtré (bleu) du potentiel de champ des cardiomyocytes 5 min après norepinephrine.

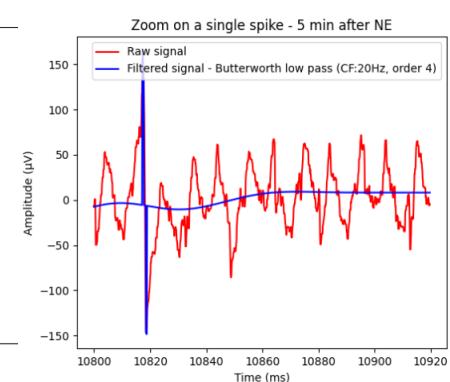


Figure 31: Zoom on Figure 30 signal - Zoom sur le signal de la Figure 30

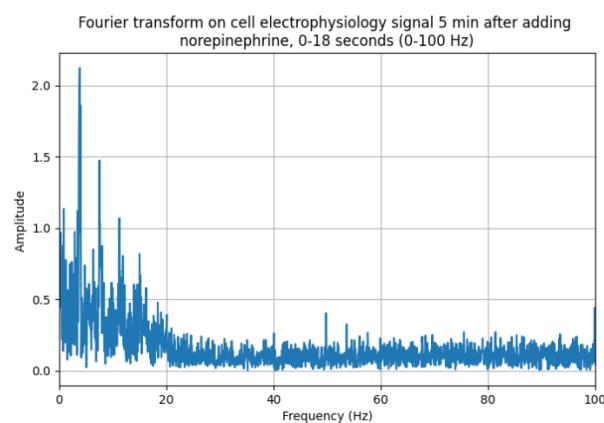


Figure 32: Fourier Transform of electrophysiology measurements 5 min after norepinephrine (0 - 18 s)- Transformée de Fourier des mesures électrophysiologiques menées 5 min après norepinephrine (0 - 18 s)

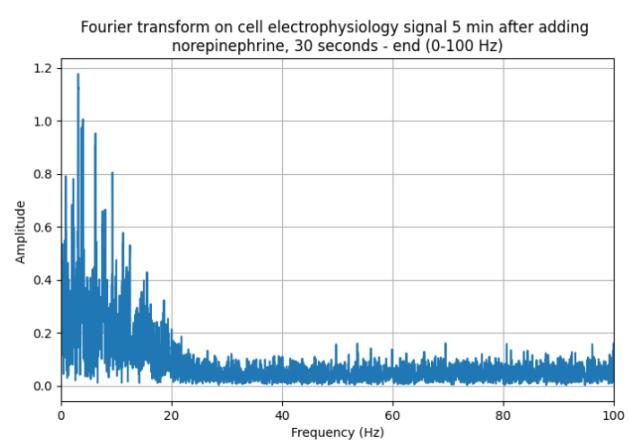


Figure 33: Fourier Transform of electrophysiology measurements 5 min after norepinephrine (30 s - end) - Transformée de Fourier des mesures électrophysiologiques menées avant norepinephrine (30 s - fin)

3 days after norepinephrine

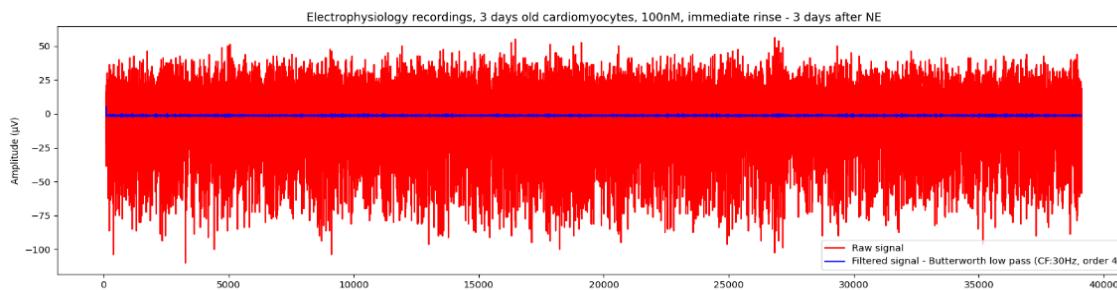


Figure 34: Raw (red) and filtered (blue) signal of cardiomyocytes field potential 3 days after norepinephrine – Signal brut (rouge) et filtré (bleu) du potentiel de champ des cardiomyocytes 3 jours après norepinephrine

The cells are not active and the signal-to-noise ratio is very low. No signal can be observed, the cells are not active and more likely to be dead (Figure 34).

Optical analysis

Optical analysis before the introduction of norepinephrine in the environment shows that the cells are beating regularly and with a relatively high mechanical amplitude⁶ (2 px) (Figure 35). The frequency of the beating is approximately **4.08 Hz**, and the periodicity of the beat is very stable, as can be seen from the Fourier graph (Figure 35) (very little noise, very pronounced main frequencies and harmonics⁷).

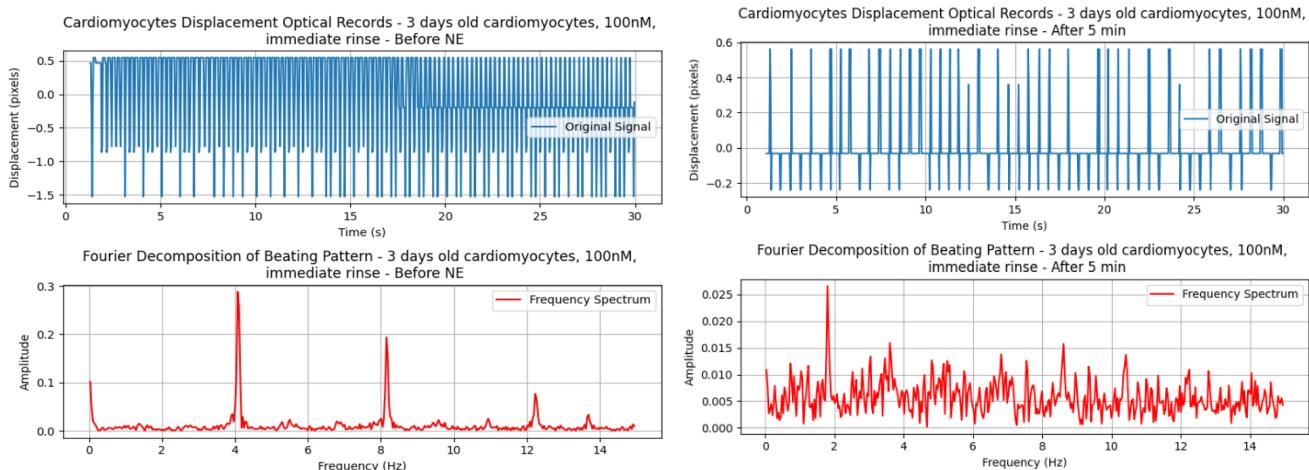


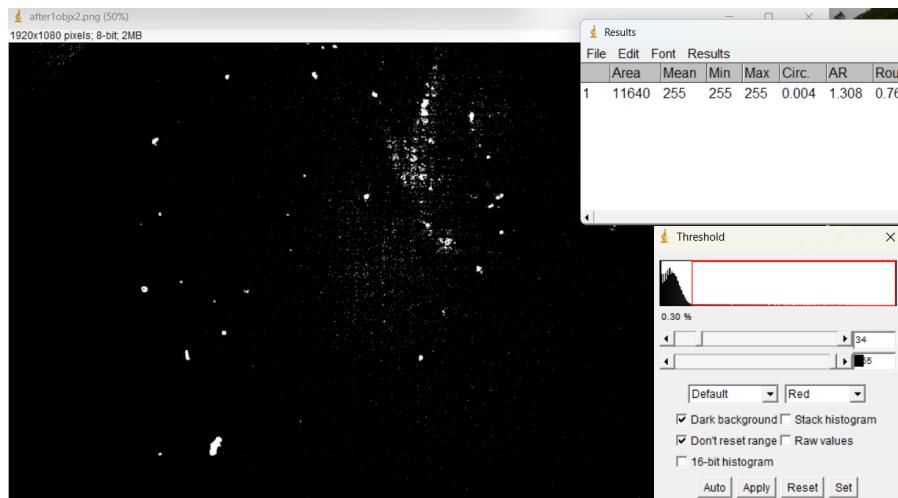
Figure 35: Optical mechanical beating observation graph and relative Fourier Transform - Before norepinephrine (left), After norepinephrine (right) - Graphique d'observation du battement mécanique et Transformée de Fourier relative, Avant la norépinéphrine (gauche), Après la norépinéphrine (droite)

⁶ The mechanical amplitude values obtained from optical observations should be treated with caution as they depend on the position of the trackers and may vary slightly depending on the position chosen.

⁷ The signal resulting from the processing of optical observations is a square-wave signal, which explains the presence of 2f, 3f, etc. harmonics.

Five minutes following the introduction of norepinephrine, the beat slows and becomes more irregular, with a frequency of 1.80 Hz. Analysis of the Fourier plot reveals an increase in noise and a decrease in the distinctness of the peaks, indicating a reduction in periodic stability. Furthermore, the amplitude of the mechanical movement of the cells during the peaks becomes less significant, with an averaged amplitude of 0.8 px per beat (see Figure 35). After a period of three days, no optical activity can be detected. The medium appears turbid on the optical records, indicating the death of a certain cell.

Fluo images analysis (3days after experiment)



- Image converted in 32 bit
- Threshold applied (same parameters for each image)
- Area fraction measured with the analyse particle tool, limited to threshold
- Fraction of fluorescent areas calculated (Fluo area/Total area)

Figure 36: Example of fluorescence image processing with ImageJ - Exemple de traitement d'image de fluorescence avec ImageJ

This analysis demonstrates that a small number of living cells persists (white dots indicates living cells whose membranes have absorbed the fluorescent dye). However, the covered area however remains minimal, indicating that the fraction of living cells is negligible (Figure 36).

General outcome (4 days after experiment)

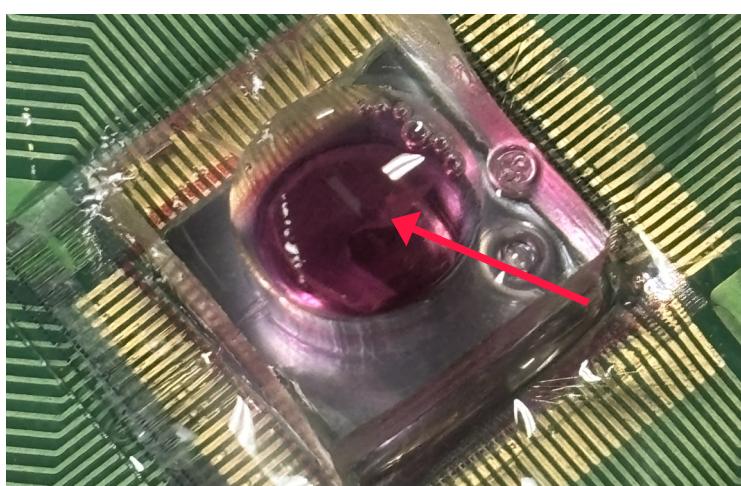


Figure 38: Cell detachment - Décollement cellulaire

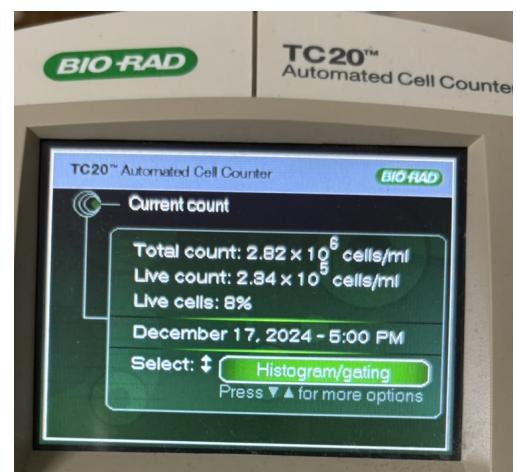


Figure 37: Cell counting (Living cells : 8%) - Comptage de cellules (Cellules vivantes : 8%)

The results of the cell counting experiment indicate that the majority of cells died, presumably as a consequence of the experimental procedure (see Figure 38). The process of cell detachment is visible to the naked eye (see Figure 37). Cardiomyocytes are known to be anchorage-dependent, meaning that cell detachment is an indication of cell death.

IV.2. Discussion

IV.3.a. Results Analysis

Exposure of 3 days old cardiomyocytes to norepinephrine induced significant changes in cardiomyocyte both optical and electrophysiological activity recordings at 100nM, followed by immediate rinsing. NE exposure led to a decreased beating frequency and amplitude as can be observed in both optical analysis (from 4.08 Hz, 2 px to 1.80 Hz, 0.8 px) and electrophysiology (from 3.97 Hz, 248.57 μ V to 3.10–3.83 Hz). Irregularities such as 10 seconds pauses could also be observed, suggesting that NE can disrupt the excitation-contraction cell coupling and as already proven by *in vivo* studies, being a precursor to arrhythmias [29].

Exposure of cardiomyocytes to norepinephrine also resulted in significant cell death within 48 h of the experiment. While this outcome aligns with apoptotic mechanisms often reported in the literature [27][28], it has to be noted that the concentration range used during the experiments (nM range) is significantly lower than the concentrations usually reported in this type of *in vitro* study (μ M range). Moreover, remaining norepinephrine should have been washed away during the washing protocol, considerably reducing the exposure time to norepinephrine. Having said that, it is still possible that the phenomena described in the literature still could have played a significant role in the induction of cell death, in particular via metabolic pathways such as β 1-adrenergic receptor activation, calcium overload, or oxidative stress [27][28], as the cells were also immature (3-5 days) which may have made them more vulnerable to external stimuli.

Nevertheless, this conclusion should be interpreted with caution, as other environmental and non-specific factors could have contributed to cellular mortality as well. For instance, mechanical stress induced by repeated rinsing and handling of the culture may have exacerbated the stress response of the cardiomyocytes. The role of these experimental artefact should be ruled out with untreated control cell culture to ensure that apoptosis is not sub-consequent to environmental factors such as contamination or suboptimal cell culture conditions.

To address these potential issues, and refine the experimental protocol, several modification could be considered, such as co-treating the cells with β 1-adrenergic receptor antagonist (beta-blockers) to confirm whether or not apoptosis is due to β 1 receptors pathway. Performing dose-response experiments, with a wide range of concentration could also permit to establish a cytotoxicity threshold.

The resultant disruption of cardiomyocytes activity and apoptosis, have permitted to highlight the probable effect of NE, even at nanomolar concentrations. These finding can also underscore the environmental sensitivity of *in vitro* cardiomyocyte cell culture, especially the immature ones.

While experimental artefacts cannot be excluded from this specific study, it remains possible that NE played a role in modulation of cardiomyocyte overall activity and state. Further studies are needed to refine the protocol and isolate the specific contributions of NE on neonatal rat cardiomyocytes in-vitro-cell cultures.

IV.3.b. Limits

Noise and filters side effects in electrophysiology measurements

During the experiments, it was observed that the metal tubes could be the source of undesirable noise introduced during electrophysiological measurements. As the tubes were too small to be connected to earth, the noise could not be reduced in these circumstances and had to be treated with filters. However, it is important to note that the use of filters can slightly modify the appearance of curves, such as amplitude, or the shape of signals, although peak preservation functions have been used to try to limit this effect.

Unpredictable basal cell behaviour

Depending on the conditions (cells outside the incubator for some time, sudden changes in environment, poor handling, contamination, etc.), it may happen that the cells are not/no longer active even before the start of the experiment. Although means are put in place before each experiment to limit this possibility (incubator on the microscope, strict protocols), sometimes the cells are not in the conditions desired for the experiments, and this can consequently compromise the results.

Uncertainties about the involvement of the device in cell death after experiments

Since the device used for these experiments is slightly different from the original device (PDMS chamber is smaller (around 0,8 mL compared to 1mL for the usual device), these changes may have therefore contributed to the cells death observed after the experiments. The smaller size of the chamber may have contributed to the faster exhaustion of the medium. This type of chamber also involves the use of metal pipes, which may interfere at some point with the medium or the cells themselves.

Exhaustiveness of the parameters tested

Due to limited resources and time (new cells every 3 weeks, limited TFT device stock, short-term internship, failed experiments), not all the parameters could be tested and analysed, and some experiments could not be repeated sufficiently. The data obtained is therefore not exhaustive, and repeating the experiment under different conditions, in particular, concentration variation, would enable more accurate results to be obtained and a better understanding of cell behaviour as a function of the influence of different parameters.

Reproducing the experiments for more trustworthy results

In the same way as for the completeness of the parameters, each experiment could not be reproduced many times due to a lack of time and resources. The results obtained therefore need to be confirmed with new experiments under identical conditions, allowing a better statistical analysis and more trustworthy results.

IV.3.c. Difficulties encountered

Contamination

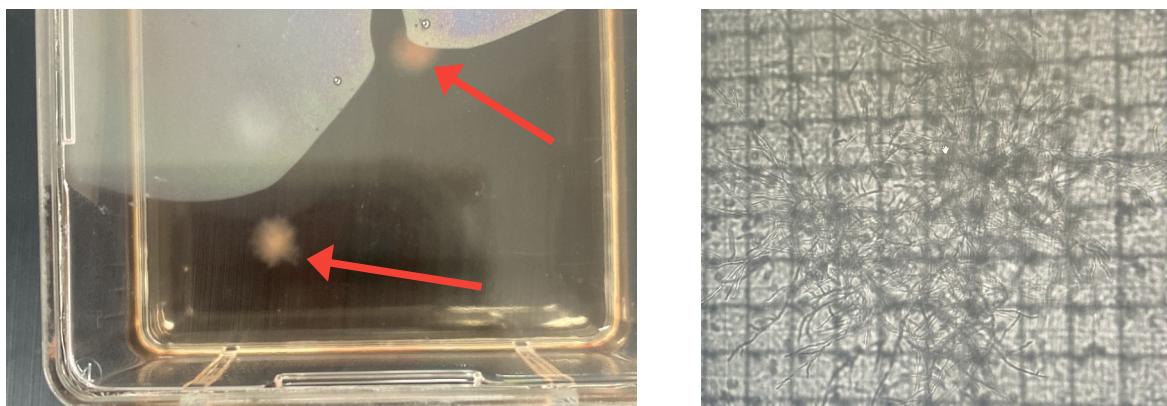


Figure 39: Example of eye-visible fungal contamination (Left), Fungal contamination under a microscope 10x objective (Right) - Exemple de contamination fongique visible à l'oeil nu (Gauche), Contamination fongique vue sous microscope objectif x10 (Droite)

One of the principal issues encountered during the course of the experiments was that of contamination, particularly that caused by fungi and bacteria during cell culture (Figure 39).

This particular type of fungal contamination has been observed to be prevalent in research facilities located in tropical regions during the summer months, attributable to the elevated humidity and temperatures that characterize these environments. Upon encountering such contamination, it is imperative to meticulously sanitise all equipment and discard contaminated cultures. This problem can lead to delays in experiments and erroneous results, in addition to the risk of cross-contamination.

Inter compatibility of equipments and reliability of the setup

The TFT multimodal platform developed in the lab remain very experimental and is used on a very small scale, no standardised equipments exists. For instance the TFT cell chamber is custom-made by cutting a squared hole in a PCB and taping the TFT technology ITO covered glass. Both are connected using wire bonding technology (see **Annex I: Ideation, prototyping and final microfluidic device used for experiments**) and secured with PDMS. The microscope used for the electrophysiology measurements must also be adapted to combine optimal cell culture conditions and optimal electronic connections to reduce noise in the signal, which often leads to complicated and not reliable setups.

Waiting time

It is well known that scientific research is an arduous undertaking, with experiments sometimes taking several days to complete and requiring daily supervision. The procurement of certain products can require a protracted period of weeks, a factor that must be duly considered for experiments conducted within a constrained reframe, such as internships or publication deadlines.

IV.3.c. Scientific contribution and perspectives

Cardiomyocytes and their behaviour have already been studied extensively in the scientific world, but most research is carried out *in vivo* or using limited *in vitro* models such as MEA, direct co-culture, or single-cell classical culture. Using the TFT multimodal platform developed in the Tixier-Mita lab offers simultaneous measurements of different physiological parameters (such as impedance, electrophysiological signals, and perhaps even optical measurements). This provides a richer overview of the response of cells to norepinephrine. By combining signals from different sensors (electrical, optical, etc.), we can analyse in greater detail how norepinephrine affects the beating activity of cardiomyocytes, as well as other aspects such as cell adhesion and contractility. This combination provides access to levels of detail and inter-parametric correlations that were previously difficult to obtain in previous *in vitro* cardiomyocyte models.

This type of study enables the lab to improve the platform, test it in real conditions, and correlate the results with other existing results. The lab's next step will be to combine co-culture of cardiomyocytes and neurones with the TFT platform and to develop a large simulation system for the heart-brain axis (Figure 40).

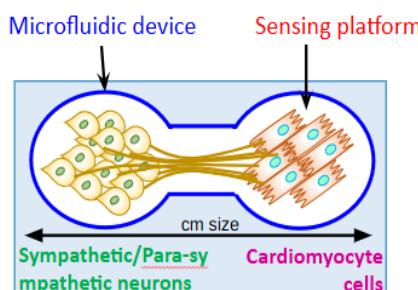


Figure 40: Scheme of the final microfluidic/sensing platform compatible with co-culture to be developed by the lab - Schéma de la plateforme microfluidique/détection compatible avec la co-culture, encore à développer par le laboratoire

V. Environmental and ethical consciousness

Throughout my internship, I was confronted with a number of ethical and environmental issues related to the scientific research field. It has become clear that the environmental impact of such research cannot be ignored, even though research is an essential area for scientific and medical progress. The field of cell culture in particular involves strict conditions of sterility, temperature and environment, which can be very energy, plastic and material intensive. Equipment such as fume hoods, incubators, centrifuges, autoclaves and so on consume a lot of energy. In addition, the sterility conditions required involve the use of a lot of single-use plastics (petri dishes, pipettes, etc.), which are themselves autoclaved and wrapped in more plastic to guarantee sterility.

Ecology in the scientific world is therefore inevitably becoming a recurrent problem, and finding the balance between scientific progress and environmental awareness is becoming increasingly challenging given the importance of addressing the current environmental crisis.

In addition to ecological considerations, ethical questions are also at the heart of the scientific field, particularly those concerning the use of animals in experiments. Although nowadays the use of animals in scientific research is limited and strictly regulated, it remains essential for certain studies. The experiment conducted in the Tixier-Mita lab use cardiomyocyte cells from neonatal rats, subsequently cultured *in vitro*. On the one hand, conducting experiments *in vitro* reduces the waste of live animals during *in vitro* studies but on the other hand, because of the tiny size of neonatal rats, collecting their cardiac cells means using many rats to obtain a sufficient sample of cells. It is therefore essential to strike a balance between the need to use animals in studies and the need to reduce the waste made on them. A way of improvement could be to use iPS cells (Induced Pluripotent cells) instead of primary cell culture for their differentiation capacity and renewal properties. However, for now this type of cell remains expensive and the cultivation protocol is a long and difficult process.

In this field, simulation seems to offer a promising new way of conducting biological experiments, providing a simple-to-use method without animal waste. However, as this is still a pioneering field, most electronic or computer simulation systems are not yet close enough to real experiments. It is in this area in particular that PM. Faure, PhD student in the lab has been working on, trying to produce an electronic platform for the electrical stimulation of cardiomyocytes.

Conclusion

This work mostly has focused on the exploration of norepinephrine-induced effects *in vitro* neonatal rat cardiomyocyte cell culture, leveraging advanced and cutting-edge of technology electrophysiological tools, in particular the TFT platform. Through various experimentations, the influence of NE at various concentrations and times of exposure on different cardiomyocyte cultures could be investigated. Parameters such as functional activity, viability or electrophysiological properties have been studied, and the outcome provided new insights into the intricate interplay between cellular activity and adrenergic signalling while exposing challenges encountered in *in vitro* modelling of the brain-heart axis.

Exposure to norepinephrine has led to disruption in cardiac activity, such as irregular periodicity and frequency modulation. Optical and electrophysiological analyses have jointly revealed an alteration in excitation-contraction coupling, which can potentially be attributed to a modification of the calcium signalling pathway. Long-term observation revealed clear cell death 48 hours after the experiments, with more than 90% of cells dying despite the rinsing steps. These results can be correlated with literature insights on norepinephrine-induced apoptosis at higher concentrations, raising interrogations about this cytotoxic effect at lower concentrations.

The use of the TFT device provided high-resolution multimodal data (electrophysiology and optical data), demonstrating its efficacy in capturing changes in signal patterns. The integration of a microfluidic system enabled the creation of a controlled environment for norepinephrine delivery and cell rinsing. This system has made it possible to choose the quantity delivered and the speed with precision while reducing the potential sources of contamination due to the repeated opening of the Petri dishes.

Despite these advances, the study also showed key limitations. The sensitivity of immature cardiomyocyte to experimental conditions, repeated contamination issues, and variability of activity between different cell cultures, could have contributed to inconsistent results and evident cell death. This work shows the importance of optimisation and rigour in experimental research. More convincing results could have been obtained by repeating each experiment many times over a long period of time.

Overall, this work contributes to the understanding of adrenergic modulation in cardiac activity by providing a base for future works to be conducted in the lab, such as neurones-cardiomyocytes co-culture. The combined use of the TFT platform and microfluidic technology presents the way forward for enhanced drug testing and physiological observations, although many adjustments still need to be made to limit artefacts and improve experimental relevance. The integration of bio-hybrid simulation systems will also enable numerous advances to be made, facilitating the reproducibility of each experiment while at the same time offering numerous ethical advantages.

This study underscores the potential of integrated systems to explore complex cellular response by bringing together fundamental research and translational application with a common goal: to better understand and treat cardiovascular diseases.

Annex I: Ideation, prototyping and final microfluidic device used for experiments

As part of the experiments I had to carry out, I've had to imagine a new microfluidic device adaptable to TFT platform to ensure the best possible distribution of neurotransmitters.

Issues faced with “classic pipette pouring method” :

- Risk of contamination of the cells culture due to opening of the lid outside of a sterile area
- Small space and lack of spatial precision when pouring
- Unwanted water movement as the drop hit the medium surface resulting in bad image resolution
- Risk of exposure to hazardous products for operator

Initial device idea :

Therefore, a semi-automated device seemed to be the best option, as the use of syringe pump to pour the solutions in the cells chamber offers a more precise flow rate and limits exposure of the cells to external environment. The use of a vacuum pump to drain the medium seemed to be necessary, due to the need of changing the medium quickly during experiments.

The initial idea was as follow (Figure 41) :

- Usage of syringe pump to control the flow rate and limit the cells exposure to external environment to prevent contamination (the pipes can operate with lid closed or inside an incubator with door closed).
- 3 Syringes filled with the neurotransmitters of interest, PBS to wash the cells and clean medium.
- Plastic pipes of known volume with metal pipes inserted at strategic points of the PDMS cell chamber connecting it to the syringes. The evacuation pipe is as low as possible to ensure proper drainages with the use of a drainage pump.

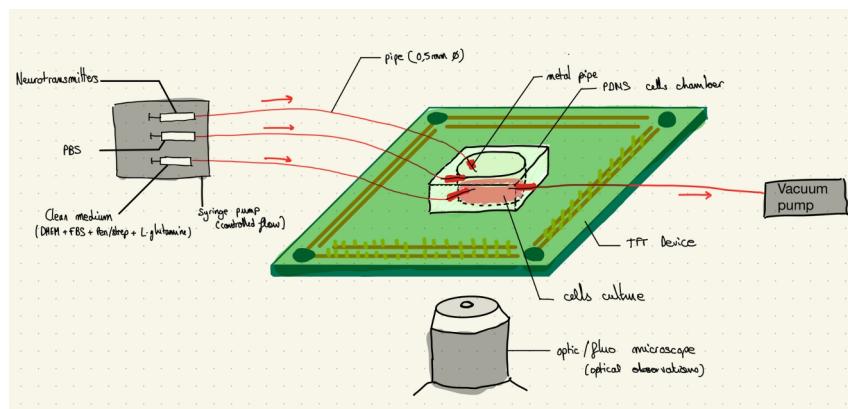


Figure 41: Scheme of the initial device idea - Schéma de l'idée initiale du dispositif

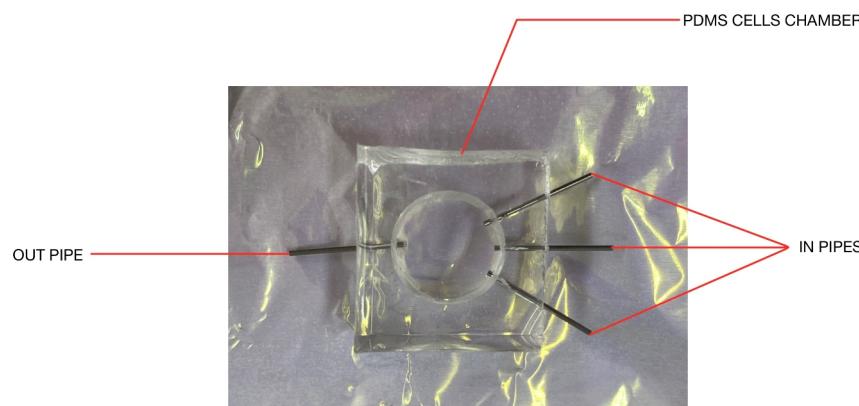


Figure 42: Final PDMS chamber – Chambre PDMS finale

The final PDMS device setup (Figure 42) was tested with the KDS 210 Legacy syringe pump and blue dye to determine the optimal flux rate. The Syringe pump was set to 5, 10, 15 and 20 $\mu\text{L}/\text{min}$ and the syringe contained blue dye. The result were filmed under the Olympus IX71 surmounted with DP74 camera, X2 objective.

The cell chamber had then to be attached to the TFT device. To prepare the TFT platform, the PCB and glass with TFT technology must first be assembled using wire bonding (Figure 43). Since wire bonding is really fragile, the connections are secured with PDMS as the cell's chamber is fixed on the device. Once the PDMS polymerised, the device is ready to be cleaned for use.

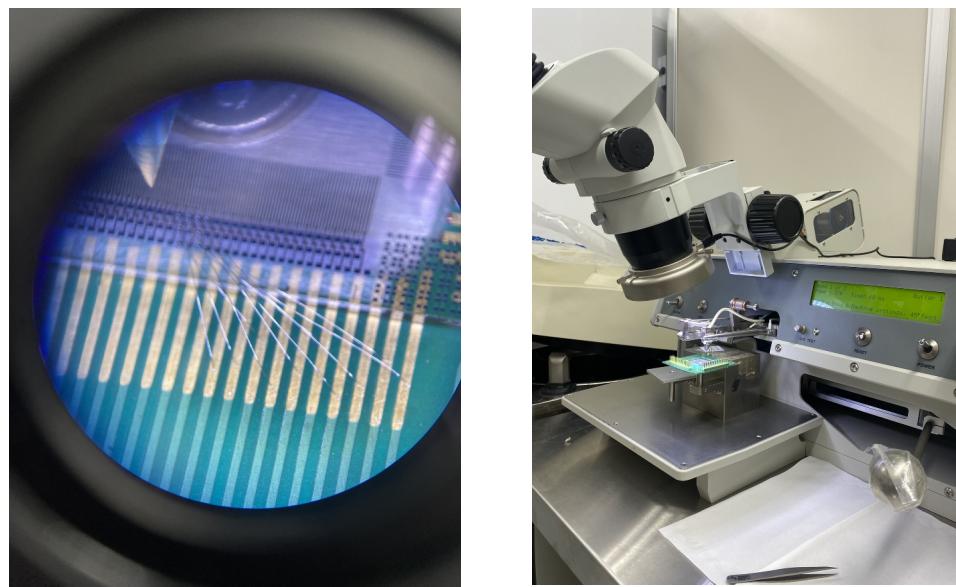


Figure 43: Wire bonding on a TFT device (Left), Wire bonding machine (Right) - "Wire bonding" sur un dispositif TFT (Gauche), Machine pour "Wire bonding" (Droite)

Annex II: Risks, precautions and individual protection measures

*This SDS for user in JP - Not correspond to the regulation of other countries.



TOKYO CHEMICAL INDUSTRY CO., LTD.

L-Noradrenaline Bitartrate Monohydrate

Revision 2
number:

Revision date: 03/04/2023

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Revision date: 03/04/2023

SAFETY DATA SHEET

1. IDENTIFICATION

Product name:	L-Noradrenaline Bitartrate Monohydrate
Product code:	A0906
Company:	TOKYO CHEMICAL INDUSTRY CO., LTD.
Address:	4-10-2, Nihonbashi-honcho, Chuo-ku, Tokyo 103-0023, Japan
Responsible department:	Global Business Department
Telephone:	+81-3-5640-8872
Fax:	+81-3-5640-8902
e-mail:	globalbusiness@TCIchemicals.com
Revision number:	2

2. HAZARDS IDENTIFICATION

Classification of the substance or mixture

PHYSICAL HAZARDS	Not classified
HEALTH HAZARDS	
Acute toxicity (Oral)	Category 3
Acute toxicity (Dermal)	Category 3
Acute toxicity (Inhalation)	Category 3
ENVIRONMENTAL HAZARDS	Not classified

Label elements

Pictograms or hazard symbols



Signal word

Danger

Hazard statements

Toxic if swallowed, in contact with skin or if inhaled.

Precautionary statements

[Prevention]

Avoid breathing dust, fume, mist, vapors or spray.

Use only outdoors or in a well-ventilated area.

Do not eat, drink or smoke when using this product.

Wash hands and face thoroughly after handling.

Wear protective gloves, protective clothing.

[Response]

IF SWALLOWED: Immediately call a POISON CENTER or doctor. Rinse mouth.

IF ON SKIN: Wash with plenty of soap and water. Call a POISON CENTER or doctor if you feel unwell. Take off immediately all contaminated clothing and wash it before reuse.

IF INHALED: Remove person to fresh air and keep comfortable for breathing. Call a POISON CENTER or doctor.

[Storage]

Store in a well-ventilated place. Keep container tightly closed.

Store locked up.

[Disposal]

Dispose of contents and container in accordance with local, regional, national regulations.

Figure 44: Extract of L-Noradrenaline Bitartrate Monohydrate SDS sheet (revised 03-apr-2023) - Extrait de la FDS du L-Noradrénaline Bitartrate Monohydrate (Révisée le 03-avr-2023)

[17]

Annex III: Cells seeding, storage and maintenance

Cells harvesting :

Since the Tixier-Mita Lab is not allowed to collect samples directly from living animal, itself, a research doctor is responsible for collecting the primary cells on newborn mice. About every two weeks, a lab member goes on the site to collect the sample. The sample is stored in a thermally insulated box filled with ice to ensure optimal cells preservation and prevent the risk of cell death. When the cells arrive at the lab, they are suspended in a **medium** solution (growth factors, nutrients and antibodies).

Cells treatment after receiving :

Since the cells are in suspension in medium, they will have sedimented at the bottom of the tube. Therefore, before counting them, they must be put back in suspension, using a vortex set to a gentle level for at least 3 minutes. When mixed, withdraw with micropipette 10 µL of the solution and place it in an ependorf tube. Then withdraw the same amount of Trypan blue, and mix it in the tube with the cell solution. Trypan Blue Solution, 0.4%, is routinely used as a cell stain to assess cell viability using the dye exclusion test.

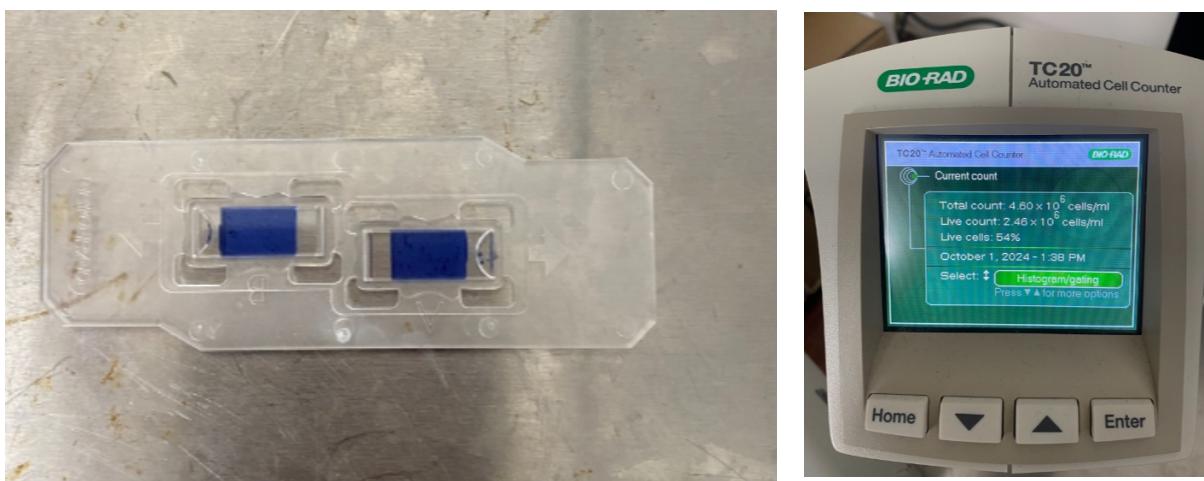


Figure 45: Cell counting plate (Left), Cell counting machine (Right) - Plaque de comptage de cellules (Gauche), Machine à compter les cellules (Droite)

Take half (10 µL) of the mixture and pour it in one of the two notches provided for this purpose on the counting plate. Do the same with the other notches. Place one side on the plate, the counting machine starts counting on its own. When the counting is finished, do the same with the other side and consider the mean of the cells number as the result (Figure 45).

The initial cell concentration is not likely to be the one we are looking for us to use during experiments. It should be around 8.10^6 cells/mL. To obtain the desired concentration, the first step is to centrifugate the given solution to separate the cells from the medium.

The centrifugation should be at low speed during a short time (300 spin/min for 5 min) to prevent any cell damage due to high speed centrifugation (Figure 46).

Precaution during centrifugation :

The centrifuge is an expensive and fragile machine, precautions should be taken during usage. When centrifuging only one sample, a weight (same weight as the sample) must be placed in the opposite location to prevent imbalance of the machine. Once the centrifugation is finished, we can observe the cell pellet sedimented at the bottom of the tube. From now on, ensure to manipulate gently the sample to avoid the pellet to mix again with the supernatant.



Figure 46: Interior of centrifuge (A), Centrifuge program (B), Sample after centrifugation (C) - Intérieur de la centrifugeuse (A), Programme de la centrifugeuse (B), échantillon après centrifugation (C)

The next step is to remove the supernatant from the tube, using a large micropipette (P1000) as the vacuum pump may damage the cells. With a simple calculation, determine the amount of the new medium that needs to be added to the cells to reach the ideal concentration (around 8.10^6 cells/mL). The cell solution is now ready to be seeded on TFT devices or stored for further use.

Cells seeding

To perform further experiments on these cells, they need to be fixed on the TFT device platform developed by Tixier-Mita Lab. As for the cell preparation, the experimental environment should be cleaned and controlled at all time (temperature, humidity ...)

To seed the cells on the device, first, collect 50 µL (or 25 µL depending on the purpose of the device and its type). Carefully place the drop on the right corner of the middle square without touching the device (which is scratch sensitive). To ensure that the drop is well placed, check under the microscope the position of the cells. When the position of the cells is satisfactory, carefully and slowly drop 1 mL of fresh medium from the corners of the cells chamber to prevent cells from moving away as it takes approximately two days for the cells to be properly fixed. Put the device in the incubator. The medium will need to be changed every one or two days.

When the cells are not immediately needed, they need to be stored to continue to grow and survive. In order to store them, gently mix the solution to evenly distribute the cells in the solution. Collect either all or half of the remaining solution (depending on the quantity left). Put it in the T-25 flask and complete with medium in order to achieve an optimal concentration of 1.106 cells/mL. Place the flask horizontally and shake gently the flask by doing an 8 on the surface of the working area so the cells can be evenly distributed on the layer. Change medium when it is starting to get lighter.

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