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Music for cells?

Rhythmic mechanical stimulations of cell cultures

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”Im Anfang war der Rhythmus”
– Hans von Bülow (1830–1894)

Abstract

This dissertation investigates how acoustic parameters and musical elements can be generated and manipulated to induce beneficial mechanical stimulations and alterations in cell cultures. The research has been conducted as part of a life science convergence environment, and the theoretical framework and experimental method in this dissertation are derived from three different disciplines: biology, music technology, and physics. The theoretical discussions centre around biological cell sensing mechanisms, the physical limitations and potentiality of audible sound to be used as mechano-acoustic cellular stimuli, and the concept of rhythm from biology and music technology perspectives. The methods include audio signal processing, physical characterisation of the experimental setup, various biological assays, and microscopic image feature extraction. Such a radically interdisciplinary approach culminated in laboratory experiments involving sound vibrations of human cell cultures using a vertical vibration system controlled by synthesized audio signals. The experimental variables included: No Vibration (NV, control), Continuous Vibration (CV), Regular Pulse (RP), and Variable Pulse (VP). The CV condition was categorised as non-rhythmic in this dissertation, while RP and VP were categorised as rhythmic conditions. The results demonstrate alterations in F-actin filament structure (length, thickness, angle) and the tendency of increased levels of cells in the G1-phase cell cycle in vibrated cell cultures. The "effect" was more apparent under the non-rhythmic (CV) condition than rhythmic conditions (RP and VP). The results also show that F-actin filament structural properties are negatively correlated ($r < -.9$), and the number of cells in the G1-phase cycle is positively correlated ($r > .9$) in relation to the magnitudes of mechanical parameters (RMS acceleration and shear stress). Nevertheless, the biological mechanism(s) responsible for the observed effects has yet to be characterised. The results from this dissertation inspire further studies on the effects of rhythmic mechano-acoustic stimulation on cellular biological rhythms (e.g., regulation of CLOCK, PER, and CRY genes).

Sammendrag

Denne avhandlingen undersøker hvordan akustiske parametere og musikalske elementer kan genereres og manipuleres for å skape positive mekaniske stimuleringer og endringer i cellekulturer. Forskningen har blitt utført innenfor rammene av et livsvitenskapskonvergensmiljø, og det teoretiske rammeverket og de eksperimentelle metodene har kommet fra tre ulike disipliner: biologi, musikkteknologi og fysikk. Den teoretiske utviklingen har fokusert på cellers mekaniske sanseapparat, de fysiske begrensningene og mulighetene ved å bruke hørbar lyd som mekano-akustisk stimulans, og forståelsen av rytmebegrepet i biologi og musikkteknologi. Metodene inkluderer lydsignalbehandling, fysisk karakterisering av det eksperimentelle oppsettet og forskjellige biologiske og mikroskopbaserte analyser. En slik tverrfaglig tilnærming ble brukt til å utføre eksperimenter som omfattet vibrering av humane cellekulturer ved hjelp av et vertikalt vibrasjonssystem styrt av syntetiske lydsignaler. Eksperimentelle variabler som ble undersøkt inkluderte: Ingen vibrasjon (NV), kontinuerlig vibrasjon (CV), regelmessig puls (RP) og variabel puls (VP). Kategorien CV var en form for ikke-rytmisk eksponering, mens RP og VP ble kategorisert som rytmiske eksponeringer. I cellekulturer eksponert for alle vibrasjonsvariablene ble det observert endringer i strukturen til F-aktin-filamentene (lengde, tykkelse, orientering), samt en akkumulering av celler i G1- cellesyklus fasen. De observerte effektene var mer markante i celler eksponert for ikke-rytmisk (CV) enn for de rytmiske (RP og VP) variablene. Resultatene viser en negativ korrelasjon ($r <$ -0.9) mellom styrken av mekanisk stimulering og strukturell organisering av F-aktin-filamenter, og en positiv korrelasjon ($r > 0.9$) mellom styrken av mekaniske stimulering og forandringer i cellesyklusfordeling. De biologiske mekanismene som er ansvarlige for de observerte cellulære effektene ble ikke karakterisert i dette arbeidet. Resultatene fra denne avhandlingen kan brukes i videre studier om effekten av rytmisk mekano-akustisk stimulering på systemer som regulerer cellers biologiske rytmer (f.eks. regulering av CLOCK, PER og CRY gener).

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Dongho Kwak
Oslo, November 2023

List of Papers

Paper I

Kwak, D., Danielsen, A., & Jensenius, A. R. "Music for cells? A systematic review of studies investigating the effects of audible sound played through speaker-based systems on cell cultures". In: *Music & Science*. Vol. 5, (2022).

Paper II

Kwak, D., Olsen, P. A., Danielsen, A., & Jensenius, A. R. "A trio of biological rhythms and their relevance in rhythmic mechanical stimulation of cell cultures". In: *Frontiers in Psychology*. Vol. 13, (2022).

Paper III

Kwak, D., Krzyzaniak, M., Danielsen, A., & Jensenius, A. R. "A mini acoustic chamber for small-scale sound experiments". In *AM'22: Proceedings of the 14th International Audio Mostly Conference*. (2022).

Paper IV

Kwak, D., Combriat, T., Krauss, S., Jensenius, A. R., & Olsen, P. A. "The effect of rhythmic vertical vibration of cell culture on the F-actin filament structure". (Will be submitted soon.)

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Chapter 1

Introduction

"There is more wisdom in your body than in your deepest philosophy."
– Friedrich Nietzsche (1844–1900)

1.1 Motivation

I have always been curious about how things work. As a child, I would often open up and disassemble my toys into the smallest possible parts and try to understand how and why they were put together in a specific way. Concurrently, I am also interested in music. I was classically trained as a flautist and percussionist. Later, I eventually pursued to combine this reverse engineering spirit and my interest in music and started my journey in music technology. As a student, I performed solo and in various types of ensembles, including duet, chamber, and orchestral music. During that time, the body awareness training I received from Professor Marth Munro at the University of Pretoria, who is known for her research and as a practitioner in embodied performance, not only improved my music performance on stage but also helped me to see the importance of the body in performance. What intrigued me the most in this context was the perception of external information.

What I found appealing was *how* one perceives information in the external world, which I thought of as a continuous signal flow. For example, what is the *input*, how is it *perceived*, and what is the *output* (Figure 1.1)? In an attempt to answer the question through the eyes of music technology, I investigated a possible interconnection between recorded music with regards to the measured loudness in dB SPL (external information/input), individual differences in auditory loudness (perception), and the impact of loudness perception on popular music consumption (action/output; Kwak, 2016). The outcome of the investigation was that there is no correlation between the loudness of the selected range of popular songs (140 singles from the top 100 Billboard Year End Chart between

INPUT ⟶ **PERCEPTION** ⟶ **OUTPUT**

Figure 1.1: Input (external information) is perceived differently by an individual, leading to a unique output.

1980 and 2010) and their commercial success. However, I became curious about what bodily factors play a role in perception.

Since the beginning of my PhD studies at the RITMO Centre for Interdisciplinary Studies in Rhythm, Time and Motion, I was introduced to the field of embodied cognition and perception more formally. Embodied perception research stresses that we get information about the things around us through our physical body (Harris et al., 2015): we use and need our body to perceive, whether through seeing, hearing, smelling, tasting, or touching.

Research in physiology has shown that the body has sensing "devices" called sensory receptors. They are largely divided into four types: chemoreceptors, thermoreceptors, mechanoreceptors, and photoreceptors (Marzvanyan and Alhawaj, 2022). These receptors have specific functions, such as sensing chemicals, temperature, physical forces, and light (Marzvanyan and Alhawaj, 2022). The dependency on these sensors for perception is revealed through physiological alterations or decline (e.g., sickness or ageing). A recent example is how COVID-19 could affect the sense of smell and taste (Santos et al., 2021). Furthermore, elderly subjects may have a degraded hearing ability primarily due to presbycusis (Cheslock and De Jesus, 2022). Such age-related hearing loss impacts how loud a sound must be before it can be perceived as useful information (von Wedel et al., 2000).

My interest and wish to learn more about the body has continued to develop during my PhD research. I have been working with a larger project called Artificial Biomimetic systems – the Niche of Islet Organoids (ABINO), funded as a UiO:Life Science convergence environment. Life sciences cover various disciplines that study the composition, structure, and function of living organisms, such as cells. The ABINO project has aimed to establish a novel technique for stem cell differentiation using mechano-acoustic stimulation to generate insulin-producing cells to treat type-2 diabetes.[1] Differentiation is a technical term used in cell biology to indicate changes or developments of stem cells into targeted cells, tissues, or organs. The starting point of the project was a curiosity about whether acoustic stimuli (sounds) could lead to *in vitro* cellular responses. *Vitro* means glass in Latin, and the phrase *in vitro* is used to denote studies outside a natural inhabitant or environment. Since the human body is made up of cells, I found this project an opportunity to get a new perspective on how the human body "perceives" external information, at the cellular level.

[1] https://www.uio.no/english/research/strategic-research-areas/life-science/research/convergence-environments/abino/

In the ABINO project, I have been working closely with biologists and physicists to understand how cells can process sounds. The focus was on the physical aspect of the cells, specifically, how mechanotransduction—the intracellular conversion process of mechanical stimulus into biochemical or bioelectrical signals—takes place in non-auditory cells *in vitro*. Consequently, my PhD project has been highly interdisciplinary, a concept and practice I will delve into more detail in different parts of this dissertation. This dissertation can be seen as part of a journey toward understanding how the body (at the cellular level) senses and responds to external changes and stimulations. This is done from my musical starting point and using music technological concepts and tools.

1.2 Research questions

The main research question of this dissertation is:

> **How can acoustic parameters (e.g., amplitude, frequency, waveform) and musical elements (e.g., harmony, melody, rhythm) be manipulated to induce beneficial mechanical stimulations and biophysical and/or biochemical alterations in cell cultures?**

My main motivation for the study has been to explore *musical* sounds, that is, audible sounds with some kind of musical structure. By audible sound, I mean sounds in a frequency range of approximately 20–20,000 Hz. Musical sounds typically consist of variations in harmony, melody, rhythm, spatiality, and timbre. For practical reasons, my experiments have focused on audible sound with little musical content. Still, the theory I have developed and the experiments I have conducted have been performed from a musical perspective. It is necessary to clarify this point to differentiate my work from projects focusing on ultrasound (in the range of megahertz), a non-audible sound type frequently used in medical fields, often without the types of sonic variations found in music.

In order to answer the main question, the following sub-questions have been investigated:

> **RQ1: How can cells sense audible sounds?**

It is not obvious whether or not *in vitro* cells can directly sense audible sounds. That being the case, it has been necessary to look into the relationships between physical sound waves in the audible range and the sensing mechanisms of the cells, which will be discussed from various perspectives in Chapter 3. Some of the fundamental theories and empirical evidence will be adduced from biology and physics to answer this question. Then, I will briefly discuss the current literature to get an overview of this subject area, which is reviewed more in-depth and systematically in Paper I.

RQ2: What parameters of musical sounds are controllable and relevant to cell cultures?

After establishing how cells can sense audible sounds, it is necessary to investigate whether musical sounds can be useful stimuli for cell cultures. Music is often complex, consisting of several layers of different variations and elements. Hence, I think it is important to examine individual musical elements to decide which are more suitable as experimental parameters or variables. Some of the intuitive parameters can be basic properties of sound, such as amplitude, frequency, and waveform. However, I have been interested in investigating this further and consider which musical element(s) is the most relevant stimulus for cell cultures. This will be discussed in Chapter 3 and further elaborated in Paper II. Furthermore, this question will be partly answered in Chapter 4, where I will discuss how and why I used music technological tools and manipulated audio signals to generate controllable parameters.

RQ3: What is the effect(s) of (musical) sounds on cell cultures?

Finally, after investigating the cellular sensing system and relevant experimental parameters based on theoretical and practical considerations, this last question will be answered through laboratory experiments. During my PhD, I have been trained to do biological lab work. This allowed me to maintain and culture cells for my experiments using the facilities at the Hybrid Technology Hub – Centre for Organ on a Chip-Technology at the University of Oslo. The data and experimental outcome relevant to this question will be reported mainly in Paper IV.

1.3 Limitations to the study

My starting point was to explore the musical stimulation of cells. That turned out to be more challenging than expected for various practical and methodological reasons.

Firstly, there was a limited amount of previous research similar to the current study. As mentioned in Paper I, there have been comprehensive studies on biological responses of plant cells to musical sounds. However, there have been only a few studies on mammal cells, specifically on human cell cultures. Such shortage meant that it was necessary to develop a basic theoretical and practical foundation combining perspectives from biology, music technology, and physics. This is one of the main contributions of my work.

Secondly, given the nature and complexity of the project, I had to prioritise characterising the experimental apparatus and environment and developing a method to measure the biological output. In addition, the same experiment was repeated several times to verify the results. Hence, I kept the experimental parameters the same and relatively simple in all the repeated experiments, which meant there was a limited amount of time to investigate musical sounds in a

broader sense. I focused on the fundamental properties of sound (amplitude, frequency, and waveform) and one musical element (rhythm). The study's conclusion might have been different if the design of the stimuli had been more comprehensive and not limited to the basic sound properties.

1.4 Contributions

This dissertation is placed uniquely between biology, music technology, physics. The theoretical framework, experimental methods, and results are an amalgam of the different disciplines that led to new ways to gain knowledge in mechanical cellular responses. The main contributions are:

- A broad literature review of previous studies encompassing biology, music technology, and physics.
- A new perspective and theoretical model of mechanical stimulation of cells encompassing biology and music technology.
- A novel coalescence of concepts, theories, and methods of biology, music technology, and physics.
- An experimental study on the effect of rhythmic mechanical stimulation (sound vibrations) on cell cultures.

In addition, the biological and music technological data produced from laboratory experiments are documented and made publicly accessible in Appendix A. I hope that the work produced and reported in this dissertation has shown the possibility of cross-fertilisation between disciplines that are often institutionally viewed as incompatible and disparate. I also hope that the novel combination of concepts, theories, and methods of different disciplines documented in this dissertation can be used as a stepping stone for future interdisciplinary research that is daring, like the ABINO project.

1.5 Dissertation outline

This dissertation consists of two parts. The first part includes chapters that introduce and contextualise my work:

Chapter 2 : This chapter begins with a discussion about the concept and practice of interdisciplinarity. The primary aim is to discuss, at the practical level, different viewpoints on how interdisciplinary research can be performed. I will also discuss some of my approaches and perspective on interdisciplinary research as a PhD candidate in the ABINO project.

Chapter 3 : This chapter, in addition to the systematic literature review done in Paper I, discusses the theoretical background for the experiments and results reported in the papers included in this dissertation with reference to

the disciplines involved in the ABINO project. I will also discuss whether musical sounds can benefit cell cultures and how they may be relevant in life sciences research. I will investigate what elements of sound and music, particularly amplitude, frequency, waveform, and rhythm, can be controlled independently to create physiologically relevant stimuli for cell cultures and to study the mechanobiological responses of the cells.

Chapter 4 : This chapter elaborates on the methodology of this dissertation. I will present a broader view of how digital signals were manipulated to control the experimental apparatus, which is discussed more in detail in Paper IV, and why I used the specific methods. I will also discuss the advantages and drawbacks of the methods used and how I plan to develop them further in the future.

Chapter 5 : This chapter summarises the results of the papers included for evaluation in the dissertation. I will discuss the findings and how the papers build on each other.

Chapter 6 : This chapter answers the research questions and discusses the research results generally. Additionally, I will indicate some paths of investigation for future research. Lastly, the interdisciplinary approach applied in this dissertation will be reflected upon.

The second part of the dissertation contains the four research papers included in the dissertation:

Paper I is a systematic literature review paper on the effects of audible and musical sounds inducing mechanical stimulation on cell cultures.

Paper II is a perspective paper focused on biological rhythms, proposing a "trio of biological rhythm" model.

Paper III is a conference proceedings paper on the construction of a mini acoustic chamber. The chamber was built to provide an environment that is acoustically controlled for small-scale acoustic experiments and measurements.

Paper IV is an experimental paper reporting on the effect of rhythmic mechanical stimulation on cells using audible sound.

Supporting materials can be found in Appendix A.

Chapter 2

A radical interdisciplinarity

”If you’re going to do interdisciplinary studies and enter someone else’s domain, [...] the least you should do is take their questions very seriously. They’ve spent a long time formulating them.”
– Waldrop, 1992, p. 150

2.1 General circumstances of the study

The ABINO project’s integration of different disciplines enabled me, as a musicology PhD fellow, to work closely with researchers from other fields (biology and physics) on a daily basis. For example, I was trained in the biology laboratories to do basic cell work, which allowed me to grow and maintain cell cultures for my experiments. The cell work included basic maintenance like changing the cell media and more advanced protocols, for example, fixing and staining of the cells, which will be described in the Chapter 4. I was also trained to acquire microscopic images of the cells and do basic analysis of the acquired images. Additionally, I have done experiments with the physicists in their labs with their guidance and listened to biophysics lectures. Some of my colleagues at RITMO referred to this approach as “radical interdisciplinarity”.

What is so radical about my project? Such an observation may come from how the distinctive disciplines—biology, music technology, and physics—are integrated into the project despite them traditionally being separated and seen as incommensurable. The Oxford English Dictionary defines a discipline as “a branch of learning or knowledge; a field of study or expertise; a subject” and, also, “a subcategory or element of a particular subject or field”.[1] At the University of Oslo, these disciplines are institutionally “double-walled” since they are further divided into three faculties: the Faculty of Medicine, the Faculty of Humanities,

[1]http://www.oed.com/view/Entry/53744

and the Faculty of Mathematics and Natural Sciences. These divisions may vary between institutions and universities, but such a tripartite separation is not uncommon in research-intensive universities. The big question was how these different disciplines could be made compatible through what seems to be "thick" academic and bureaucratic walls.

The lack of communication between disciplines and faculties has been pointed out before by Charles Percy Snow (1905–1980) in his popularised book called *The Two Cultures and the Scientific Revolution* (Snow, 1961). Even 40 years after that, Snow's observation remained virtually the same when Wenzel (2001, p. 525) wrote:

> It is generally known that communication between natural scientists, on the one hand, and social scientists and humanities scholars, on the other, is not always the best. Open conflict commonly arises when the latter groups take the natural sciences and technology as a research area. Here it is not unusual that, despite initial good will and shared views, the two groups eventually turn against each other.

However, the communication gap seems to be narrowing in more recent years. Massey (2019, p. 69) writes that the apparent division between so-called scientists and humanists is outdated, and "there has been a substantial increase in interdisciplinarity within science." He asserts that scientists are "more willing and capable of studying across other areas" and "humanists have become more sophisticated in their use of technology" (Massey, 2019, p. 69). This means that the two cultures now utilise and share common or similar technological tools and knowledge (Massey, 2019). So, according to Massey, the development and use of technology in both cultures have contributed to bridging the communication gap.

The ABINO project can be an ideal example of Massey's observation of a smaller disciplinary communication gap through more willingness of "scientists" to embrace multidisciplinary approaches and more sophisticated use of technology by "humanists". As a so-called convergence environment, the ABINO project has aimed at putting together a multidisciplinary team to work towards a common goal. Thus, in terms of interdisciplinary research, the focus of the discussion should now move beyond Snow's notion of the communication gap. To that end, I find it more useful to think about what it really means to *do* interdisciplinary research. The follow-up questions that I asked came at different levels:

1. High level: What does interdisciplinarity mean?

2. Low level: How does one do interdisciplinary research?

3. Practice: What are the practical aspects of doing interdisciplinary research?

4. Reflection: Does one really acquire new theoretical and/or empirical knowledge and innovative solutions through such a "radical" approach?

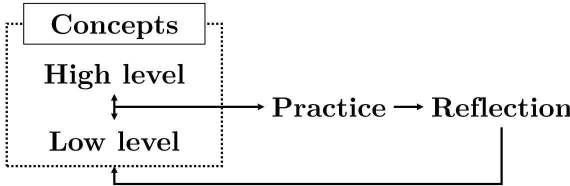

Figure 2.1: A continuing flow of concepts contemplated at high and low levels, put into practice, and reflected that helps to concretise and develop the concepts further.

I would like to imagine these levels as shown in Figure 2.1 where the consideration of concepts at both high and low levels is applied and put into practice, and it is reflected upon. Subsequently, the reflection helps to reconsider, or possibly update, and concretise the concepts.

Given the above, I will examine the theoretical and practical aspects of the questions I had about doing interdisciplinary research in an attempt to position myself optimally or strategically between several disciplines combined through the ABINO project. The first three questions (high level, low level, practice) will be dealt with in this chapter and the last one (reflection) in Chapter 6 while referring back to this chapter. A good starting point would be to do some groundwork: establish a definition and description of interdisciplinarity as the foundation of this dissertation.

2.2 Interdisciplinarity: What and how

According to the Merriam-Webster dictionary, interdisciplinary is when "two or more academic, scientific, or artistic disciplines" are involved.[2] The practice of involving two or more disciplines is not a novel idea. For instance, Pythagoras's idea and application of mathematics in studying harmonics while considering metaphysical (ethical) aspects of music involved several "disciplines" (Barker, 1990). What is more, the first use of the term interdisciplinarity is known to be in the 1920s already (Frank et al., 1988). Since then, although it has been a century, it is generally accepted that no definition can succinctly describe what interdisciplinarity really means (Holbrook, 2013).

The practice of interdisciplinarity often begins from one's "desire to see things whole" (Frank et al., 1988, p. 146) as "the wholeness of each system is more than the sum of its elements" (Wenzel, 2001, p. 529). In line with that, Jensenius (2022, p. xvii) suggests interdisciplinary research uses "real synthesis of approaches". Interestingly, the emphasis is on the *real* synthesis. This is to differentiate interdisciplinarity from other disciplinarities, such as multidisciplinarity, referring to when "different disciplines working together,

[2] https://www.merriam-webster.com/dictionary/interdisciplinary

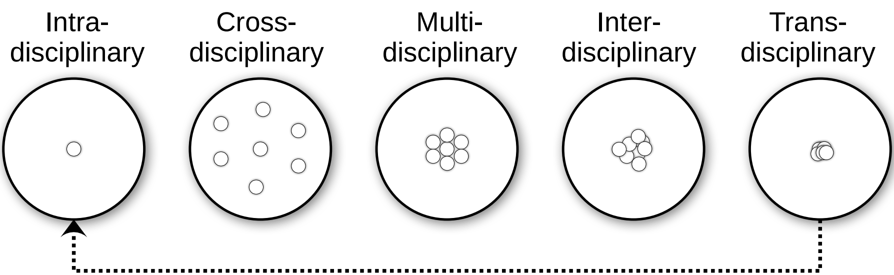

Figure 2.2: Continuously "evolving" disciplinarities (Jensenius, 2022, p. xviii).

each drawing on their disciplinary knowledge" (Jensenius, 2022, p. xvii). Such distinction at a high level is important, but how does the synthesis take place?

The committee on Facilitating Interdisciplinary Research defines interdisciplinarity as the following (of Medicine, 2005, p. 2):

> Interdisciplinary research is a mode of research by teams or individuals that integrates information, data, techniques, tools, perspectives, concepts, and/or theories from two or more disciplines or bodies of specialised knowledge to advance fundamental understanding or to solve problems whose solutions are beyond the scope of a single discipline or field of research practice.

The essence of this definition is again synthesis and integration, but also to generate *new knowledge* or *solutions*. Through the definition, the committee provides several clues about how to do interdisciplinary research, such as how many disciplines and what to integrate.

On a side note, and somewhat confusingly, when a project is "problem-oriented research" that is "beyond former division of faculties" to generate a new solution that is impossible for one discipline to develop by itself, the approach is sometimes referred to as transdisciplinary (Mainzer, 2011, p. 278). According to this notion, interdisciplinary communication is merely a stage to get to such transdisciplinary research (Mainzer, 2011).

This idea is reflected on Jensenius's loop of evolving disciplinarity (Figure 2.2; Jensenius, 2022, p. xviii). Jensenius (2022, p. xviii) presents the levels in a sequence as how disciplinarity can evolve from intradisciplinary, multidisciplinary, crossdisciplinary, interdisciplinary, transdisciplinary, and back to intradisciplinary. According to this model, a genuine internalisation of different disciplines should be considered transdisciplinary. This is similar to the notion that interdisciplinarity is a step that leads to transdisciplinary research (Mainzer, 2011).

The point of the loop model is not to say that transdisciplinarity is the ultimate goal or the best out of all the other types of disciplinarity. Also, it is not necessarily true that every interdisciplinary project would produce a transdisciplinary outcome, whether that outcome has to do with a method, data, researcher, or research field. However, by having an overview of different

levels, I think a researcher or research field (project or discipline) can know how to position oneself optimally and strategically in a project that involves many different disciplines.

James Britt Holbrook (2013) presents more practical descriptions of interdisciplinarity. An interesting viewpoint of Julie Thompson Klein that Holbrook emphasises is that different disciplines are considered as different ways of communicating information, and he views the issues arising in interdisciplinary communications as a "linguistic problem". This approach provides some more perspectives on how to do interdisciplinary research. He discusses three types of interdisciplinary communications (Holbrook, 2013, p. 1869–1876):

- Habermas-Klein thesis: The integration of two languages in order to generate a common understanding

- Kuhn-MacIntyre thesis: The notion of incommensurability of different disciplines and the necessity to learn a new discipline as a "second-first" language

- Bataille-Lyotard thesis: The importance of failure of interpretation and/or communication and the invention of a new language that follows

These proposed theses are not only descriptions that reveal diverse approaches within interdisciplinarity but also offer some practical suggestions or advice. For instance, Holbrook goes further than the idea of integrating two or more disciplines. The theses that he proposes point to the fact that communication may even break down, resulting in incomprehensible or untranslatable dialogues between disciplines. In those instances, he makes a case that other disciplines' language must be learned or a new language must be created to solve a problem at hand (Holbrook, 2013).

What may be interesting to consider at this point is Noam Chomsky's notion of language. Chomsky regards language as a system of thoughts and not merely for communication, asserting that the externalisation of language is minimal compared to what is internalised (Chomsky, 2008, p. 357). Benjamin Lee Whorf also noted that language "informs thinking" (Subbiondo, 2005).

These notions bring another perspective to interdisciplinary communication. If we consider the idea of language as an "instrument of thought" (Asoulin, 2016) in the context of interdisciplinary communication, there are deeper underlying aspects to be reflected upon than the definitions given by the committee on Facilitating Interdisciplinary Research and Holbrook's theses; perhaps it is implicit in their definitions. Then, the issue is not solely about integrating theoretical and methodological principles and communication between disciplines. Instead, the primary issue becomes whether one can adopt a whole new way of thinking. This could mean that individuals or disciplines genuinely internalise different disciplines' concepts, theories, and methods. Having done that, Holbrook's notion of interdisciplinary communication will eventually follow from the embodiment of a new discipline(s) (i.e., a new system of thoughts).

Whether this is possible and whether such internalisation is still interdisciplinary at the level of individual researchers and disciplines will be discussed later in Chapter 6. For now, the following section will discuss a more practical aspect relevant to interdisciplinarity.

2.3 Cooperation versus collaboration

At a more practical level, the differences between cooperation and collaboration may help to illustrate a critical factor to consider when doing interdisciplinary research. Roschelle and Teasley (1995, p. 70) distinguish cooperation and collaboration as the following:

> We make a distinction between 'collaborative' versus 'cooperative' problem solving. Cooperative work is accomplished by the division of labour among participants, as an activity where each person is responsible for a portion of the problem solving. We focus on collaboration as the mutual engagement of participants in a coordinated effort to solve the problem together. We further distinguish between synchronous and asynchronous activity. Although we do not propose that collaboration cannot occur in asynchronous activity, we focus on face-to-face interactions, which can only occur as a synchronous activity.

What does this all mean? Let us take a practical example. Usually, different departments in a company work towards a common goal (e.g., providing quality services and products, revenue generation, and business success). When different departments (e.g., production, marketing, sales) are *cooperating*, the departments are working in their separate departments. In order to achieve the common goal, they are each given an individual goal(s) and set up their own strategies to achieve it. If a department achieves its own goal, the other department would not be rewarded and share the accomplishment of the goal, although it may benefit from it indirectly, depending on the circumstances. It would be the same case when a department fails to achieve its goal. The other department will not be punished for the failure. However, it may be indirectly affected by the failure because it might mean that the company will struggle to survive, for example.

On the other hand, when different departments are *collaborating*, it could mean that employees from different departments are put in a newly created department to work towards a common goal(s). The employees in this environment are put in the same "space" to strategise and generate a solution(s) together. Once there is an agreement, they may cooperatively share the workload. It could also mean that they are put in the same physical office space to achieve a more "synchronous activity", although whether we need a physical office to work has been questioned since the COVID-19 pandemic (Coff et al., 2022). When departments collaborate, goals, accomplishments, and failures are truly shared than when they merely cooperate.

The company example differs from the academic research setting, but it can be fairly similar at the operational level. When different disciplines cooperate, not only the accomplishment or failure is not shared between other disciplines involved in the project, there are no real “agreed methods and solutions” between the participating disciplines (Roschelle and Teasley, 1995). The situation can be comparable to the collection and juxtaposition of disciplines where each discipline maintains its traditional identity (i.e., multidisciplinarity; Holbrook, 2013). Therefore, it does not mean collaboration happens just because different individuals from different departments or disciplines are put together as a group.

A missing key element for genuine collaboration might be the “Joint Problem Space” (Roschelle and Teasley, 1995). The “Joint Problem Space” is “a negotiated and shared conceptual space” through a “framework of shared language, situation, and activity; not merely inside the cognitive contents of each individual’s head” (Roschelle and Teasley, 1995, p. 70–71). In the “Joint Problem Space”, active interdisciplinary communication can occur, and the accomplishment or failure of the goal is truly shared (Holbrook, 2013; Roschelle and Teasley, 1995). As it is noted by Roschelle and Teasley (1995, p. 70), “collaboration is a coordinated, synchronous activity that is the result of a continued attempt to construct and maintain a shared conception of a problem.”

Taken together, an agreed goal, method, and solution through *persistently active* negotiations and discussions that are *coordinated* between disciplines in a shared abstract and physical space are crucial factors in doing interdisciplinary research. In the following section, those interdisciplinary concerns and views will be discussed together with some of the fundamental questions concerning all three disciplines involved in the ABINO project.

Chapter 3

Interdisciplinary considerations and perspectives: A dialogue between biology, music technology, and physics

"Dialogue illuminates what people are not saying."
– *Robert Towne*

3.1 Introduction

Effects of music on humans, both psychological and physiological, have been studied since ancient times (Nilsson, 2008). Even in the present day, evidence supporting that music is beneficial for the well-being of humans is accumulating in various fields of modern research (Welch et al., 2020). Notably, one of the emerging interdisciplinary research areas involving studying the effects of musical sound is life sciences, particularly cell biology. There are two main underlying motivations in the literature. Firstly, since the human body consists of cells, they are exposed to musical sounds as much as humans, which is the point that tries to cover the ecological validity (Kumeta et al., 2018). Secondly, the potential beneficial effects of mechanical stimulation—sound waves are mechanical—on cell cultures have been largely overlooked (Goetzke et al., 2018).

The experimental cell models are typically not *in vivo* (i.e., cells in their natural environment, such as the human body) but *in vitro* (i.e., cell cultures in an artificial microenvironment in a biology laboratory, including Petri dishes, cell plates, or specially designed chips). Broadly, there have been two approaches: 1) playing selected pieces of the recorded music of various genres and 2) playing

simple tones, such as a sine tone. The first approach poses challenges since complex stimuli, such as music, consisting of numerous elements (e.g., dynamics, harmony, melody, rhythm, structure, texture, and timbre), make it difficult to establish causal relationships between the experimental stimuli and biological changes observed in cell cultures. Additionally, reproducibility may suffer since there may be variations of, for instance, different performances, recording and mastering techniques, and instruments between different versions of the recordings of the same piece of music. For similar and other reasons, performing live music to cell cultures in biology laboratories would be impractical. By contrast, the second approach can provide a degree of control and better reproducibility by using mathematically precise sinusoidal waves at a specific frequency and amplitude for a certain amount of time. However, one could question how many people listen to such sounds on a regular basis, even though listening to sine tones is accessible with synthesizers.

My PhD project's main aim has been to investigate and develop parameters of musical sound—in the form of mechanical stimuli—that are biologically relevant to cell cultures using music technological concepts and methods. It can be questioned what music or music technology could contribute to life sciences.

In light of this, an interesting viewpoint could be that music is a multi-layered arrangement of patterns, and its "coming to life" depends on the patterns and their continuous interaction unfolding spatially and temporally. Such an idea that constituent parts sum to a whole echoes a concept of organicism in the musicological context. Beard and Gloag describe the concept as the following (Beard and Gloag, 2005, p. 124–125):

> Organicism reflects the idea that the musical work is an organism in which parts combine together as a functioning whole, appropriating the body as a metaphor for the musical work.

The idea of organicism seems to be traceable back to the ancient philosophical period (e.g., in the periods of Plato and Aristotle; Solie, 1980). Later, Georg Wilhelm Friedrich Hegel (1770–1831) articulated the idea, particularly on aesthetics and works of art: "if the work is a genuine work of art, the more exact the detail, the greater the unity of the whole" (Beard and Gloag, 2005, p. 125). This notion existed for "the purpose of validating a certain body of works of art" (Kerman, 1980, p. 315) that was used to illustrate an artwork to be excellent (Solie, 1980), especially during the eighteenth century among those who advocated tonal music (Street, 1989) and German instrumental music (Kerman, 1980). Some of the notable exponents of organicism include Heinrich Schenker (1868–1935) and Rudolph Réti (1885–1957), who, in their musical analyses (Solie, 1980), aimed at "demonstrating organicism" through revealing the "organic unity" or "inner coherence" of a piece of music composition (Kerman, 1980).

Organicism has not been without criticism due to its ideological tendency towards only uplifting "truly great" tonal music (Beard and Gloag, 2005; Kerman, 1980). Kerman (1980, p. 331) noted that "what is important is to find ways

of dealing responsibly with other kinds of aesthetic value in music besides organicism."

For the purpose of this dissertation, I will take the concept out of this musicological-ideological context and borrow its way of thinking about music. For instance, from a music theory perspective, different musical elements (parts), such as harmony, melody, and rhythm, are arranged to work in tandem to create music (the functional body). For that reason, as a corollary argument to what I have discussed earlier in this chapter, music can be a complex yet fascinating system to compare with a biological system in that many different sub-components work spatially and temporally in tandem to constitute a functional system.

Historically, music and biology were not as divorced as it seems through the eyes of modern academic divisions. Peter Pesic (2022, p. 1–2) notes that the understanding of music as part of the Pythagoreans' quadrivium (arithmetic, astronomy, geometry, and music), known as the liberal arts, had a significant impact on the development of physical and mathematical sciences and biomedicine. For example, through important figures such as Galen (129–216), Andreas Vesalius (1514–1564), René Descartes (1596–1650), and Robert Hooke (1635–1703), the human body was understood through music and sound, such as fibre vibration theory, which is apparent in Vesalius' *Epitome* (1543), Descartes' *Traité de l'homme* (1664), and Hooke's *Micrographia* (1665) (Pesic, 2022, p. 109–115). The centuries' worth of efforts and investigations of sonic features of the human body led to the development of some of the most vital diagnostic tools for studies around auscultation (i.e., listening to body sounds through a stethoscope), pulse, and ultrasound (Pesic, 2022, p. 2). Leopold von Auenbrugger (1722–1809) and René Laennec (1781–1826), for instance, were educated both in physiology and music. They "treated the body as a musical instrument" and studied "sonic diagnostics" through auscultation (Pesic, 2022, p. 151).

In spite of this particular connection between music and biology, simply playing a piece of music to cell cultures might not be the most effective experimental approach. As an interdisciplinary strategy crossing over to life sciences, music may become more relevant and interesting when its elements are taken apart and considered individually. Such an approach may provide control over each element separately and, if necessary, the capability to reconstruct different elements back together.

This begs the question: how do we discern what musical elements are relevant to cell biology? It is not obvious how cells would physically respond and differentiate distinct qualities of musical elements, or any other audible sounds for that matter. Therefore, along with the discussion about the interdisciplinary concerns and views, the aim of this chapter is to investigate the relevance of musical elements at the cellular level. Before moving on, it is also necessary to clarify that I am not trying to study music's "therapeutic effects" on the body.

With this in mind, the following sections comprise a theoretical overview of the problems faced when correlating musical sounds as stimuli with cellular responses and seeking out the potential usefulness, relevance, and benefits of

musical elements as parameters of mechanical cellular stimuli.

3.2 Can cells "perceive" sound?

In life sciences research, such as cell biology, biotechnology, tissue engineering, and regenerative medicine, mimicking *in vivo* conditions is a critical research problem in generating cells that could ultimately be used for treatments or drug testings for various diseases (Segeritz and Vallier, 2017; Selvakumaran and Jell, 2005). In order to do that, the cell cultures are carefully maintained and engineered in biology laboratories using numerous biochemicals and biomaterials as external stimuli (Badekila et al., 2021). How do cells sense these external chemical and physical stimulations?

3.2.1 Biology: What is a cell?

The word cell is derived from the Latin word *cella* meaning a small room,[1] and it is generally known that cells are the most fundamental forms of life. Whether single (e.g., bacteria) or multicellular organisms (e.g., plants or animals), cells have similar functional and structural features: a cell membrane, cell wall, cytoplasm, and nucleoid or nucleus (O'Connor and Adams, 2010).

Cells are generally divided into two kinds: prokaryotes and eukaryotes (Cooper, 2000; Figure 3.1). Prokaryotic (pro- means before and kary- means nucleus) cells usually refer to rod-shaped bacteria that can be about 1–10 µm in diameter. From the outside inwards, prokaryotes have a cell wall, plasma membrane, and cytoplasm. The most distinguishing feature of prokaryotes from eukaryotes is that they lack a nucleus, as the name suggests, but it has a nucleus-like feature called the nucleoid. Typically, the nucleoid does not have a nuclear membrane and is not separated from the cytoplasm (Cooper, 2000).

On the other hand, eukaryotic (eu- means true) cells refer to plant or animal cells that can size between about 10–100 µm. Eukaryotes are structured with the plasma membrane (some have cell walls such as plant cells), cytoplasm, nuclear membrane, and nucleus. They are typically a more complex form of life than prokaryotes, having compartmentalised organelles within the cytoplasm and filament networks called the cytoskeleton. The cytoskeleton is generally responsible for the mechanical properties and activities of the cells, for instance, the shape and movement of the cells. Eukaryotes usually function in aggregates, whereas prokaryotes can function independently as single-cell organisms (Cooper, 2000).

Human cells are essentially complex eukaryotes. It is estimated that there are about 200 different types of cells (Cooper, 2000) and about three trillion cells in the human body (Bianconi et al., 2013). These cells have specialised cellular components that connect the intracellular to the extracellular environment; thus, they can respond and adapt to the changes in their external microenvironment.

[1] https://www.merriam-webster.com/dictionary/cell

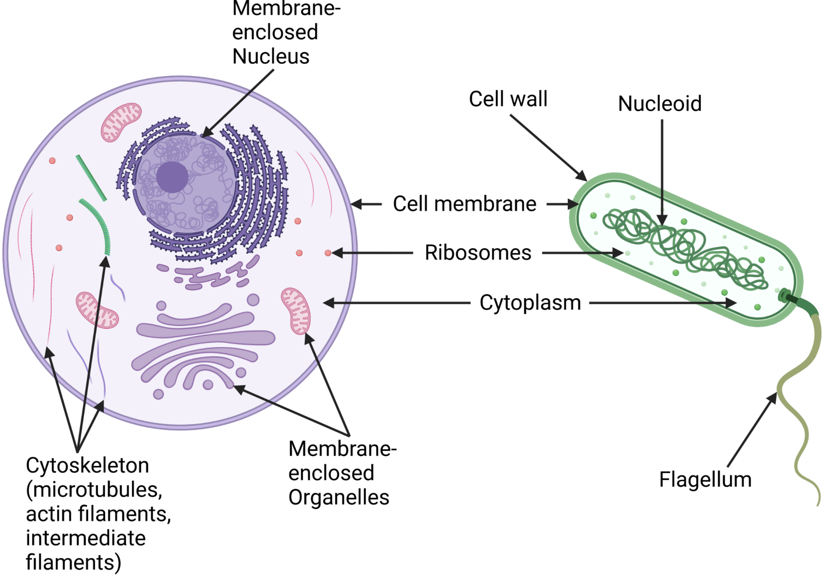

Figure 3.1: A simplified illustration showing components of eukaryotic (left) and prokaryotic (right) cells. Created with BioRender.com.

3.2.2 Biology: The sensors of the cells

Cellular responses to external stimulations can be intriguingly complex as the cells consist of a large number of molecules, such as various types of proteins, acting as “agents”. The process of cellular sensing mechanisms mostly starts from a sensing “device” called receptors. They are proteins that are located either inside the cells (intracellular or cytoplasmic) or in the membrane of the cells (transmembranal; Miller and Lappin, 2022). The signals that the receptors receive are small molecules called ligands which can be divided mainly into hydrophobic (intracellular ligands) and hydrophilic (extracellular ligands) types (Miller and Lappin, 2022). Hydrophobic ligands can pass through the lipid cell membrane and bind to intracellular receptors, whereas hydrophilic ligands can only bind to the extracellular side of transmembrane receptors (Miller and Lappin, 2022).

The binding of ligands to specific receptors creates signals that can trigger intracellular biochemical processes by activating or deactivating proteins in a specific order (intracellular signalling cascades) that ultimately leads to changes in cellular functions, such as cell growth, migration, or death (O’Connor and Adams, 2010). Considering this fact, the supposition that musical sounds or sine tones can positively impact cell cultures becomes absurd; how could sounds possibly affect the biochemical processes of the cells?

Cells have additional types of receptors sensing not only chemical stimulations

by ligands but also mechanical stimulations (Nair et al., 2019). Since sound waves are mechanical, the type of receptor that would be specifically relevant is mechanoreceptors. Cells experience different types of physical forces, such as compression, tension, and shear, in their natural environment. These mechanical signals contribute to regulating the biological processes of the cells (Mohammed et al., 2019). Moreover, the cells can form biomechanical memory, resulting in faster and stronger cellular responses to recurrent mechanical perturbations (Thompson et al., 2020).

3.2.3 Physics: What about the physical properties of sound and cells?

It seems plausible, then, that sound waves generated from playing musical sound through a speaker could induce relevant mechanical forces upon the cell cultures. The effect of such mechanical forces could interact with the cells and may change the state of motion of the cell or change the shape of the body of the cells that are viscoelastic. The cells could sense these mechanical interactions and changes through the mechanoreceptors. Nevertheless, two basic physical limitations directly deny this plausibility. Firstly, the physical wavelength of any audible sound is extremely large compared to the size of a cell. A wavelength (λ) can be defined as:

$$\lambda = \frac{v}{f} \tag{3.1}$$

where v is the velocity (m/s) and f is the frequency (Hz). The frequency and the wavelength are inversely related. If we take the shortest wavelength within the audible frequency range, thus the highest audible frequency of 20 kHz, at the temperature of 20°C when sound travels at about 343 m/s, for example, the wavelength would be approximately 1.7 cm. In comparison, the size of human cells is within micrometres. Therefore, the pressure change at the cellular level would be homogeneous, which means there would be no physical deformation of the cells. Additionally, since cell cultures are generally maintained in a liquid (e.g., cell culture media), the speed of sound in water ($\sim$ 1480 m/s) is different than in air at the same temperature. This means the wavelength of the same frequency will be even longer ($\sim$ 7 cm) than when sound travels in the air.

Another consideration is that sound playing from a speaker has to travel through air before moving into the cell culture medium. So it is necessary to take into account how the acoustic impedance difference between air and liquid limits the amount of transmission before the sound waves can reach the cells. The acoustic reflection coefficient (R) is defined as:

$$R = \left(\frac{Z_2 - Z_1}{Z_2 + Z_1}\right)^2 \tag{3.2}$$

where Z is the acoustic impedance of a medium which is defined as:

$$Z = \rho c \tag{3.3}$$

where ρ is the density (kg/m^3) of a medium and c is the speed of sound (m/s) in the same medium. The acoustic impedance of sound waves travelling in air at the temperature of 20°C is $Z_1 \approx 413\,\mathrm{Pa} \cdot \mathrm{s/m}$, and in water is $Z_2 \approx 1.5\,10^6\,\mathrm{Pa} \cdot \mathrm{s/m}$. Thus, the reflection coefficient between air and liquid will be extremely high ($R \approx 0.9989$); sound waves would be reflected almost entirely (∼99 %) on the boundary between air and liquid. Theoretically, only about 1 % will be transmitted. That is to say, the amount of sound energy that “hits” the cells would be physically restricted.

In essence, although it is possible that cells can sense external mechanical forces through mechanoreceptors, the physical differences and limitations of audible sound compared to the cells are too great to be overlooked. Thus, from a physics perspective, it is difficult to argue why audible sounds should have an effect on cells. So if the claimed cellular responses to musical sounds are to be true (Exbrayat and Brun, 2019), there must be something else that the *in vitro* cells can sense when sounds are played to them.

3.3 Cells “catching the next wave”

The scrutinisation of what *in vitro* cells are sensing when audible sounds are played to the cells could go in several directions. One is to consider that longitudinal waves (sound waves) are converted into transverse waves (surface waves) in the Petri dish. Recently, a trending research topic has been to show the effects of surface acoustic waves on different cell lines, using specially designed cell plates with mini electrodes and a function generator, where ultrasounds were used to induce surface waves (Brugger et al., 2020; Imashiro et al., 2021; Rufo et al., 2022).

There are two reasons why it is widely supposed that cells can directly sense such surface waves. Firstly, the wavelength of the induced surface waves falls within the micrometre range that is comparable to the size of the cells. Thus, there is more chance of physical interaction between the waves and the cells, possibly causing mechanical deformations of the cells (Bonakdar et al., 2016).

Secondly, the basic cell cultures are generally designed for the cells to attach to the bottom surface of the Petri dish or cell plate, which is known as the 2D cell culture system. In contrast, a so-called 3D cell culture system, such as when cells are suspended in the cell media or cultured in hydrogel materials, is also used under specific conditions and for different applications (Kapalczynska et al., 2018; Madl et al., 2018). The surface where the cells can attach is sometimes treated with an additional layer consisting of different types of proteins (e.g., fibronectin; Ramsey et al., 1984) to create the microenvironment (i.e., extracellular matrix (ECM)) enabling broader types of cells to attach (J. Huang et al., 2021). This extra layer is usually negligibly thin, and the surface waves can be induced or progressed through such layers (Imashiro et al., 2021).

3.3.1 Biology: Cell attachment mechanisms

Cells can be divided into either adherent or suspension types. For example, most human cells are adherent cells (Tseng and Santra, 2022, p. 193). This means they are "anchorage-dependent" and, in cell culture, they need to attach to a rigid surface to grow (Stoker et al., 1968). Suspension cells, on the other hand, can be "free-floating" in cell media (Tseng and Santra, 2022, p. 193–194). Another critical ground for cells' possible sensing of ultrasound surface waves is how cells can become attached to the surface of a cell culture environment, such as a Petri dish.

Usually, when adherent cells are put onto a Petri dish, they are spherical in shape and sink down to the surface. Then, the cell surface adhesion process commences, predominantly mediated by the interaction (chemical bond) between proteins located in the cellular membrane and proteins in the ECM (Jansen et al., 2015). A common type of such anchoring junction is the focal adhesions that are mediated by integrin proteins on the cell membrane (Cooper, 2000).

The adhesion process mainly occurs through the conformational changes, which can occur in nanoseconds to seconds (Bu and Callaway, 2011), of integrin ectodomain (i.e., a part of the protein that extends out to the extracellular environment from the membrane; Jansen et al., 2015). By the time the cells are fully attached, which can take a few minutes to several hours depending on the cell type, the cells generally start to flatten while spreading over the surface (Derakhti et al., 2019).

The involvement of integrins in cellular attachment to the surface of the Petri dish or the ECM is one of the key elements in understanding how the cells sense mechanical stimulations like surface waves in 2D cell culture systems. This is due to the fact that integrins have another critical role, which is sensing extracellular mechanical stress (Mohammed et al., 2019). The integrins also join the intracellular structures, such as F-actin filaments, to the extracellular structures (Sun et al., 2016).

Therefore, cells could sense the surface waves with comparable wavelengths through integrins, which are one of the critical pivot points between physical intracellular and extracellular environments (Mohammed et al., 2019).

3.3.2 Physics: Vibrations and fluid flow

Audible sounds could also induce a transverse type of surface wave, but it is still not obvious what the cells might be sensing. As we considered already, the wavelength would be too large. In addition, the sound produced by a loudspeaker would cover a large area in the cell plate, while ultrasound transducers produce acoustic waves that are more focused. Take the displacement of the bottom surface of a cell culture slide only in the vertical direction, for example, in Figure 3.2. Consequently, the vibrational modes generated in the cell plate caused by audible sounds would consist of complex patterns that are larger than that of ultrasounds, which will make it difficult to correlate with cellular responses.

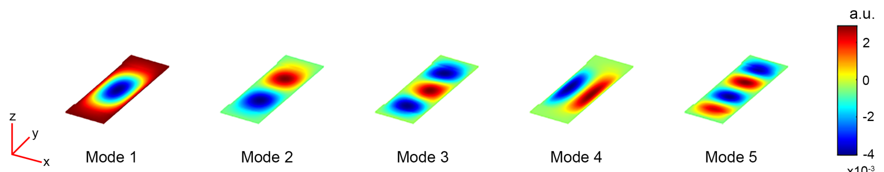

Figure 3.2: The first five z-displacement modes of the bottom surface of a cell culture plate (25.5 x 75.5 mm^2, without the walls) when vibrated vertically using a 50 Hz sine tone.

However, an ascertainable fact can be deduced through the vibrating cell plate. When the bottom surface and the walls of the plate vibrate or sympathetically resonate with the sound waves, the resonance could perturb the cell media (liquid) and induce movement (Jeong and Kim, 2015), creating a fluid flow within the wells of the cell plate. The disturbances can be at the liquid surface (e.g., Faraday waves) but also in the bulk of the liquid, which is a phenomenon known as streaming flows (Perinet et al., 2017). The acoustic-induced streaming depends on the amplitude, frequency, and height/depth of the liquid in the wells (Guex et al., 2021). This makes it possible to think that cells can be mechanically stimulated by the shear stress generated by flowing fluid in the cell plate (e.g., wall shear stress created by the resistant force from the surface of the cell plate when fluid flows next to the surface).

3.3.3 Biology & Music technology: Physiology of the human auditory system (the ear)

Let us pause for a moment here and think about the natural ways that biological systems, such as humans, sense and process sound waves. How do we humans sense and perceive sound? This is possible in a few different ways.

For example, sound can be felt through the body, especially at lower frequencies. To give an illustration, imagine sounds produced by an array of large subwoofer speakers at a live concert. Similarly, persons with impaired hearing can learn to differentiate distinct sounds through induced vibrations on their skin (Perrotta et al., 2021). Sound can also be perceived through bone conduction, for instance, “the mechanical vibrations of the bones of the skull that can propagate to the inner ear” (Bartel and Mosabbir, 2021, p. 4). This is an essential mechanism for bone-anchored hearing aids developments (Mudry and Tjellstrom, 2011) and has more recently also been introduced in commercial headsets for music listening.

In general, a person with a healthy hearing ability would perceive (audible) sounds entering through the outer ear of the auditory system, which is a subject area also relevant to music technology. We could compare the mechanism of the auditory system to the alternative mechanism discussed above to gain some insights from the natural biological process. The alternative mechanism is that

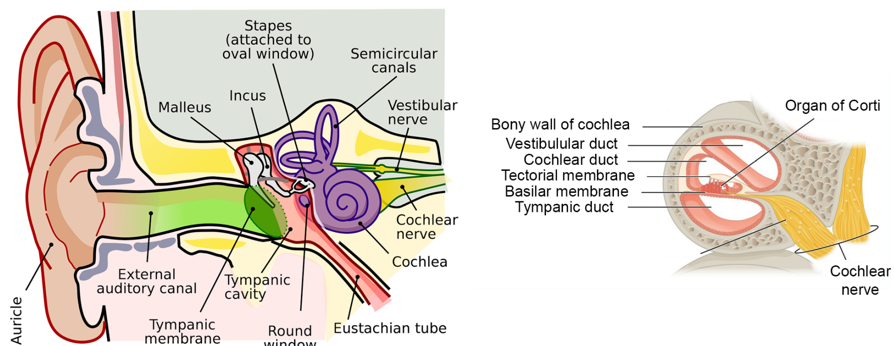

Figure 3.3: (Left) An illustration of the human ear anatomy. The colours green, red, and purple show the outer, middle, and inner ear, respectively. Image: Chittka and Brockmann (2005), distributed under CC-BY 2.5 license. (Right) A cross-section showing the anatomy of the cochlea. Image (adapted): Openstax, distributed under CC-BY 4.0 license.

cell cultures are more likely to sense the fluid movement in a vibrating cell plate induced by sound waves rather than direct sound waves.

The human auditory system is an intricate and fine structure that specialises in transforming the information from mechanical signals (sound waves) into electrical signals (nerve impulses)—the process known as mechanotransduction—that is sent to the brain (Gillespie and Muller, 2009). The system is largely divided into three sections: the outer ear, middle ear, and inner ear (Figure 3.3). When sound waves enter the outer ear, the tympanic membrane (the eardrum), which separates the outer and middle ears, moves sympathetically to the sound waves (Gillespie and Muller, 2009). The movement is transferred to the three bones known as the ossicles, consisting of the malleus, incus, and stapes, in the middle ear (Gillespie and Muller, 2009).

The mechanism of the tiny ossicles in the middle ear is particularly crucial for mechanical energy conservation through a type of lever effect (Campbell and Tan, 2022). Such effect is achieved by the connection between the tympanic membrane (a large surface area) and the footplate of the stapes covering the oval window on the cochlea (a smaller surface area) through the ossicular coupling, where a large mechanical advantage can be gained (Mansour et al., 2019, p. 93). In effect, the middle ear can amplify the mechanical signal received by the tympanic membrane, which can be up to $\sim$34 dB (Mansour et al., 2019, p. 93). This amplification is nonlinear (less or no amplification when incoming signals are high in intensity; known to be a protective mechanism) and extremely frequency discriminatory (Gillespie and Muller, 2009).

It is interesting to note at this point that hair cells, the key mechanoreceptor in the auditory system, are located in the cochlea filled with special fluids—endolymph and perilymph (Wangemann, 2006)—in the inner ear. More specifically, the hair cells are found in the organ of Corti coupled to the basilar

membrane in the *scala media*, known as the central cochlear duct, filled with endolymph (Figure 3.3). The cochlear duct is situated between the other two ducts called *scala vestibuli* and *scala tympani*, known as the vestibular and tympanic ducts respectively, and they are filled with perilymph.

A pump-like movement of the ossicles attached to the oval window—an opening on the cochlea that is sealed with a flexible membrane—leads to perilymph moving through the vestibular duct and to the tympanic duct, generating a standing wave in the perilymph. Although perilymph (liquid) would not be compressible, such a movement is possible thanks to another opening of the cochlea called the round window that can be found at the other end of the tympanic duct. The round window is also covered with a flexible membrane, which can be displaced by the initial mechanical movement from the oval window (Wangemann, 2006).

During this process, the cochlear duct coupled to the two other ducts through two different membranes—the Reissner’s membrane separating the vestibular and cochlear ducts and the basilar membrane separating the cochlear and tympanic ducts—is displaced according to the standing wave at a certain frequency in the perilymph. The main mechanism for such displacement is the transmission of the wave propagation in the perilymph to the endolymph causing a specific section of the basilar membrane that separates the two liquids to vibrate sympathetically. As a result, the organ of Corti is displaced together with the hair cells. Simultaneously, the hair cells are bent in different directions (depolarised or hyperpolarised) due to viscous force (Billone and Raynor, 1973) and to shearing action of the tectorial membrane in the cochlear duct as a mass load on the hair cells (Richardson et al., 2008; Zwislocki, 1988). To put it concisely, the waves that travel through the perilymph and endolymph cause the hair cells to be displaced between two different types of membranes, and the physical bending of the hair cells is an important initial step triggering intracellular signalling to generate nerve impulses.

Consequently, by the time the information of the sound vibration reaches the inner ear, the signal is preprocessed by the outer and middle ears to match the incompatible impedance between air and liquid (Mansour et al., 2019, p. 214–215). Additionally, sound waves can travel through the air cavity by the acoustic coupling in the middle ear, which can reach the inner ear. However, this mechanism is considered to be insignificant (Merchant et al., 1997). The hair cells primarily sense the fluid’s sympathetic movement to the sound waves in the cochlea, not direct sound waves through the air.

The auditory system’s key mechanism of mechanotransduction unveils the natural need for the mechanical energy transformation from sound waves into fluid waves that can lead to mechanical deformation and shear stresses on the hair cells (Billone and Raynor, 1973). This information makes it even harder to presuppose that audible sounds will directly affect non-auditory cell cultures in the lab. It would be more feasible to think that the cells are mechanically stimulated through the fluid movement by the mechanical vibration or resonance of the cell plate induced by the sound waves. In fact, the auditory system could even be suggested as a model for mechano-acoustic stimulation of the cells.

Therefore, an investigation of the fluid flow in the chambers of cell plates when the cell culture is exposed to sound stimuli in experiments may be critical.

Taken together, although the beneficial effects of musical sounds on cell cultures are a highly intriguing subject area, it is necessary to come down to a lower and more practical level to clarify what might be the biological and physical mechanism(s) at the cellular level. Some of the issues with regard to the experimental paradigm of mechano-acoustic stimulation of cell cultures have been elucidated by examining interdisciplinary concerns and views. The theoretical consideration revealed the limitations (e.g., the physical size differences and the extreme acoustic impedance mismatch) but simultaneously provided new possibilities worth further investigation (i.e., the vibration of cell culture plates that leads to the fluid moving and may generate shear stresses at the cellular level). While keeping this in mind, it is also necessary to examine what musical elements might be useful and relevant as experimental parameters to study mechanical cellular responses in addition to the basic physical elements of sound (i.e., amplitude, frequency, and waveform).

3.4 Relevant sound and music features in the context of cell biology

In this section, I will start the discussion briefly with the aforementioned basic sound parameters (amplitude, frequency, and waveform). At the cellular level, changes in the amplitude of sound—correlates to a sound's perceived loudness—could determine how much sound pressure level a cell or cell culture can endure. Moreover, the amplitude can affect the temperature of the cell media. For example, Kumeta et al. (2018) reported the temperature increase of ∼0.4°C in the cell media when a sine tone was measured to be played at 94 dB SPL. Such variation can be significant since cells can respond sensitively to temperature. For instance, cell growth and death rates can be negatively affected if the temperature is higher or lower than 37°C in biological incubators, which is similar to the temperature range of the human body (Leber et al., 2012; Watanabe and Okada, 1967).

The sound frequency—what is perceived as a sound's pitch—is inversely related to the physical wavelength. Hence, the higher the frequency, the shorter the wavelength, and the higher the chance of sound waves directly interacting with the cells. Conversely, the lower the frequency, the longer the wavelength, and the lower the chance of direct effects on the cells, but the higher the chance of sound waves directly interacting with larger structures than a cell, such as a Petri dish.

As far as the waveform is concerned, many experimental studies have utilised pure (sine) tones as stimuli. These are sinusoidal waves at a fixed frequency, as shown in Paper I. Other basic waveforms, such as sawtooth, square, or triangle, have been used at least once by Kumeta et al. (2018) but did not show any significant effects on the cells compared to sine tones.

The three sound parameters (amplitude, frequency, and waveform) describe the "static" properties of a sound. Together, they can be sufficient to develop a contactless technique to physically control and stimulate cell cultures as an alternative to biochemical stimuli. For example, cells can be sorted and aggregated using acoustic tweezers techniques or by creating the Faraday wave in a Petri dish (Guex et al., 2021).

However, most natural sounds (including musical) have a temporal development, making them more "dynamic". In pursuing my main research goal, I wanted to explore more musical parameters. While considering the interdisciplinary discussions above, one important question to the current study was if any other parameters are overlooked but as essential as the three physical elements of sound. Subsequently, I encountered an interesting and striking claim that caught my attention: "*rhythm* is a fundamental phenomenon in all physiological systems" (Haken et al., 1991, p. v, the emphasis added).

3.4.1 Music technology: What is rhythm?

As a classically trained musician, the word rhythm strikes me as a technical term. It reminds me of one of my music professors when I was a music student who used to tell me "you must feel the inner beat between the beats". One of the rhythmic exercises he illustrated was to swing an arm like a metronome and count different rhythm patterns in numbers out loud. Keeping an accurate subdivision of the beat in time was one of the many ways that I experienced rhythm, but it is broadly a more fundamental concept and phenomenon.

Hans von Bülow (1830–1894), a German conductor and pianist, remarked, "in the beginning was rhythm" (Cowan, 2011). The statement is not an exaggeration, as it is hard or probably not possible, to imagine music without rhythm. The essentialism of rhythm has been debated in the musicology field, and there are academic contentions regarding whether rhythm is the most important element in music. For example, some argue that there can be "music without a clear meter and pulse, such as in free time music, but not without rhythm" (Dowling and Tighe, 2014, p. 93). It is not in the scope of this dissertation to discuss that part (e.g., rhythm vs meter) of the rhythm theory thoroughly, but what can be relevant and interesting to note is that strict regularity does not seem to be a requirement for rhythm. As will be discussed further below, different degrees of regularity, as opposed to complete randomness, is an important factor in rhythm.

Curiously, rhythm and its "essentiality" are not unique to music. Many years before Haken et al. (1991) noted the rhythmicity in physiological systems, Hermann Muthesius (1861–1927), a German architect, stated that "rhythm characterizes every human activity; it is the first law of every expression of our being" (Cowan, 2011).

Rhythm functions as an important element that organises sound in music, but it provides structure and increases cost-effectiveness in many other human activities beyond music: architecture, visual art and design, poetry, sports, and so on.

In the field of architecture, rhythm plays a vital role through the "social effects of human-driven spatial concerns" (Prieto De La Fuente, 2018, p. 18). This is also based on economic concerns, such as reinforcing the roof with fewer materials at an interval while maintaining the necessary forces and strength to support the roof and the building without collapsing. In design, rhythm is an important factor that creates movement and style (Chan, 2012). In poetry, rhythm gives structure and enhances the emotional experiences by creating "variety and energy" (Carper and Attridge, 2020, p. 10). In sports, rhythm and temporal structure in mind can help to stabilise and enhance sports performances (MacPherson et al., 2009).

This generalised function of rhythm could indicate that the signals in time and space that we perceive as rhythmic are a consequence or manifestation of an efficient and/or optimally functioning system or structure. Such a broad description of rhythm is also echoed by the RITMO application written by Danielsen and Jensenius[2]:

> We define rhythm as ordered patterns in time. Such patterns are not static, because they are made up of both repetition/periodicity and variation/expressivity. Rhythm as a phenomenon thus ultimately relies on the complementary nature of these two poles, as articulated by the notion of rhythm as a 'changing same' (LeRoi Jones). Rhythm patterns emerge when we perform or observe repeated events, and they can occur in any sensory modality (visual, auditory, etc.) or even in combinations of modalities (audiovisual, etc.). The capacity to mentally 'form' rhythmic time structures out of such changing events is profoundly human and probably species specific, and it conditions many aspects of human life and expression.

The outspread notions of rhythm suggest that rhythm is not limited to only a small handful of disciplines, and it could be defined as such. Consistently, Danielsen and Jensenius[3] define rhythm as "ordered patterns in time", which can be more inclusive across different disciplines.

Having said that, one could question: why should ordered patterns be in time? A possible origin of the word rhythm, *rhythmos*, a Greek word meaning "to flow", could give an interesting point of view to such a question (Danielsen, 2022).

Flow can be both a mental and physical state. To cite an instance in psychology, Mansfield et al. (2012, p. 327) writes that flow is "a pleasurable performance-enhancing psychological state," and "the high degree of absorption and concentration evident when an individual is engaged in an activity".

Physical flow is studied primarily in the fields of fluid mechanics (White, 2006, p. 1–4). This specific area of study provides some physical elucidations of why things—soft solids, liquids, or gases—can deform and flow, including "spatial variations of temperature" or "variations in concentration" of a matter (Guyon,

[2] https://www.uio.no/ritmo/english/about/index.html
[3] https://www.uio.no/ritmo/english/about/index.html

Source	Definition
Macmillan[4]	A regular pattern of sounds, movements, syllables, or in nature
Merriam-Webster[5]	Movement, fluctuation, or variation marked by the regular recurrence or natural flow of related elements
Oxford Learner's Dictionaries [6]	A strong regular repeated pattern of sounds or movements
Wikipedia[7]	A regular recurrence or pattern in time
Wiktionary[8]	A regular quantitative change in a variable process

Table 3.1: Some general definitions of rhythm

2015, p. 5). For instance, the state (smooth/laminar or fluctuating/turbulent) of the flow can be characterised by knowing the Reynolds number. This can be determined by flow velocity, fluid density, fluid viscosity, and linear (geometric) dimension. The higher the Reynolds number, the more turbulent the flow may be (White, 2006, p. 5).

What is perhaps more closely connected to the word *rhythmos* within the ancient Greek context could be Heraclitus's (c. 535–475 BCE) notion of "everything flows" (Savenije, 2015), which is, possibly only later, referred to as *Panta Rhei* (Barnes, 2002, p. 49). In connection with the notion of *Panta Rhei*, Plato (c. 428–348 BCE) paraphrased by saying that "everything moves and nothing rests" (Barnes, 2002, p. 49). Their observations are true in a way that even minuscule particles, that of Brownian motion, for instance, constantly move due to thermal motion. Particles move more at a higher temperature than at a lower temperature and, theoretically, eventually stop moving at the lowest temperature limit of the thermodynamics, known as the absolute zero (Guyon, 2015, p. 13–14). Even in materialistic things that appear to be solid and stationary, there is still space for atoms to move; atoms are brought so close to each other strongly that they vibrate (Guyon, 2015, p. 5).

Whether it is a mental or physical state, flow unfolds in *time*. Savenije (2015) noted that flow is used metaphorically to say that "everything [both material things and observers] changes continuously" over time. Thus, flow can indicate some movement and constant change of something (e.g., stream) in time (Danielsen, 2022). In that respect, flow's relevance to time is implicit.

What does rhythm have to do with flowing, then? Similar to flow, rhythm is also not static but a constant variation(s) in time. More fundamentally, flow and rhythm are both a "temporal phenomenon" (Danielsen, 2022). For example, "music is an art of time", which is expressed with and manifested through rhythm that generates movements (Alperson, 1980). More generally known definitions of rhythm, as shown in Table 3.1, also illuminate this feature of rhythm.

From these definitions, key concepts and terms that seem to be important for rhythm include:

- Variability
- Repeatability
- Regularity
- Pattern
- Time

In a single sentence, to describe rhythm more comprehensively, these keywords could be restated as "a series of events that regularly varies and repeats, generating patterns in time".

Last but not least, Danielsen (2022) interestingly notes that rhythm is an experience by a perceiver when encountering a signal, who can give a structure to the signal that unfolds in time, suggesting that rhythm exists in time and the perceiver and not in the materials as such. So, the physical signal that flows is one thing and a perceiver who senses and perceives the signal unfolding in time is another. But they are interrelated and can exist together. Rhythm, then, "emerges" when the flow of signal unfolding in time is perceived and given a perceivable structure or form (Danielsen, 2022). Additionally, Bengtsson and Gabrielsson (1983, p. 28) describe rhythm as "a response to certain kinds of sound sequences and Danielsen (2006, p. 46–47) denotes that "rhythm is conceived as an interaction of something sounding and something not sounding". In fact, this has been pointed out by several other scholars (see Clarke (1985), Kvifte (2004), and Johansson (2017)). Due to this, the regularity of a series of signals or events and intervals between them play a critical role in rhythm perception. Bartel and Mosabbir (2021, p. 3) writes the following:

> When there are more than 16 events per second (about 62.5 Hz) cognitive event fusion changes from individual event perception to hearing pitch frequencies. The rhythmic stimulation effect on cells, however, continues to be related to the events per second even, for example, at 40 or 60 events per second [which is equivalent to 40 or 60 Hz]. If the events are slower than about 10 s per event [which is 0.1 Hz], long-term memory may allow perception as functional units of musical form but these will not be heard as individual events.

Therefore, rhythm perception can be dependent on the duration of the silences or the distances between events. It is interesting that the "perceivable" rhythmic temporal scale of the "silences" between events can be broader (shorter

[4] https://www.macmillandictionary.com/dictionary/british/rhythm#rhythm___9
[5] https://www.merriam-webster.com/dictionary/rhythm
[6] https://www.oxfordlearnersdictionaries.com/definition/english/rhythm
[7] https://en.wikipedia.org/wiki/Rhythm
[8] https://en.wiktionary.org/wiki/rhythm

Rhythm level	Description
Ultradian rhythm	Recurring cycles completed more than once per day ($<$ $\sim$24 hours)
Circadian rhythm	Recurring cycles completed daily ($\sim$24 hours)
Infradian rhythm	Recurring cycles lasting longer than a day ($>$ $\sim$24 hours)

Table 3.2: Three different temporal levels of chronobiology

and longer) at the cellular level. In biology, relevant functional rhythms can be measured from a tiny fraction of a second to years (Reppert and Weaver, 2002). For instance, the baroreceptor and chemoreceptor control systems can sense large intervals of the Mayer waves in the arterial blood pressure, which can be smaller than 0.1 Hz ($\sim$10 seconds), and be functionally entrained at that frequency (Julien, 2006). This kind of cycle can be found in many biological systems and is referred to as biological rhythms, which will be discussed in the following section.

3.4.2 Biology: Biological rhythms

Rhythm, as an ability that is mostly idiosyncratic to humans and possibly to other species (Fitch, 2013), is used to realise signals and events that are spaced at fairly regular durations and intervals in biology. Through categorising patterns of biological events in time, useful information about the system can be revealed.

The first scientific publication related to the study of biological rhythms is known to be a short observational report by a French geophysicist and astronomer Jean Jacques d'Ortuous de Mairan (1678–1771), which is known to set the interest in investigating the rhythms in biology in motion (Kuhlman et al., 2018). In *Observation botanique* (de Mairan, 1729), de Mairan reports that a mimosa pudica plant, which is a heliotrope, meaning the leaves always follow the source of light, the rhythmic opening and closing of leaves of the plant continued without the external photic stimuli.

Eventually, the studies of rhythmic phenomena in many different biological and environmental systems have been conducted and reported, including fluctuations of weather and water levels, recurrences of diseases and earthquakes, and changes in the abundance of animals (Dewey, 1970). Most of the reported biological rhythms were entrained to the light and dark (or day and night) cycles. This is known as diurnal cycle driven by the earth's rotation resulting, for example, in temperature and humidity fluctuations over the land surface (Betts, 2015). There have been studies around non-photic stimuli, such as meal times, physical activities, and noise levels, but they can still be strongly related to the light and dark cycles (Reinberg and Ashkenazi, 2003).

Biological rhythms can be largely divided according to three different temporal levels of chronobiology (Table 3.2; discussed more in-depth in Paper II). In the human body, these biological rhythms, especially circadian rhythms, are

regulated by the so-called master clock (i.e., the suprachiasmatic nuclei; SCN), which consists of about 20,000 neurons located in the hypothalamus of the brain (Reppert and Weaver, 2002). The SCN, in mammals generally, is known to be the only location in the body that can receive external light-dark cycle information through the retinohypothalamic tract pathway, get entrained, and regulate the timing of biological processes at other locations of the brain and different tissue levels throughout the body (Reppert and Weaver, 2002).

Rhythmic phenomena can be observed even at the level of cells. Cellular rhythms are mainly regulated by a self-regulatory ∼24-hour transcription-translation feedback loop (TTFL) at the molecular level (Yang et al., 2017). Transcription in cells refers to the deoxyribonucleic acid (DNA) transcription process, where a part of the encoded DNA information is copied onto the molecule called ribonucleic acid (RNA). The RNA information is written in “the same language as it is in DNA”, hence the name, transcription (Alberts et al., 2002). The translation is essentially a post-transcription process that occurs in the cytoplasm (outside the nucleus) where RNA information gets “decoded” to produce a specific type of amino acid chain that subsequently is assembled into a protein (Alberts et al., 2002).

Mechanisms for TTFL started to be uncovered in the late 1970s when Ronald J. Kanopka (1947–2015) performed an experiment on Drosophila (fruit flies) and discovered alterations in behavioural periodicity in Drosophila with induced gene mutation of PERIOD (PER) (Handler and Konopka, 1979). TTFL is a negative loop mainly driven by four clock proteins, including ARNTL (more commonly known as BMAL1), CLOCK, CRYPTOCHROME (CRY), and PERIOD (PER) (Gallardo et al., 2020; Partch et al., 2014; Yang et al., 2017). The CLOCK:BMAL1 heterodimers (i.e., protein dimers with two different protein monomers) in the nucleus promote the DNA transcription to produce PER and CRY genes (Partch et al., 2014). Subsequently, PER and CRY heterodimerise (PER:CRY heterodimers) in the cytoplasm and move into the nucleus, where the dimer acts as a suppression factor to inhibit the transcription of CLOCK:BMAL1 (Partch et al., 2014). The PER:CRY heterodimers are eventually degraded by the ubiquitin system, which removes proteins intracellularly (H. J. Guo and Tadi, 2022), thus leading to reinitiation of CLOCK:BMAL1 transcription (Partch et al., 2014). This self-regulated cycle is approximately 24 hours (Partch et al., 2014).

The rhythmic activity of these so-called clock genes is typically analysed using bioluminescence (meaning “living light,” the light emission from living organisms; Kricka, 2005) reporter gene assay techniques (Doruk et al., 2020). A biological assay refers to “the process by which the activity of a substance is measured on living material,” or non-living in *in vitro* models (Schachter and Pirmohamed, 2012). The most commonly used bioluminescence reporter protein is luciferase (a light-producing enzyme) which is found in fireflies and Renilla reniformis (also known as sea pansy).[9] Thus, when the luciferase reporter gene is activated, through the chemical bioluminescence process, light is emitted

[9]https://no.promega.com/resources/pubhub/enotes/bioluminescent-reporter-genes/

from the reporter, and the brightness of the luminescence can be measured live. Accordingly, fluctuating levels of gene activities, for example, ARNTL, CLOCK, CRY, and PER, can be measured and quantified over time.

Interestingly, mechanical cues (alterations) at the cellular level can have an impact on the regulation of the circadian rhythms in the cells. For instance, Yang et al. (2017) used mouse mammary cell explants as a model to illustrate the correlation between microenvironment stiffness and cellular rhythms. When the cells were grown on a stiff 2D culture plate, as opposed to in softer 3D matrigel cell culture conditions, the measured amplitude of the circadian rhythms (∼24-hour CLOCK:BMAL1 expression rhythm) in the cells was suppressed. This mechanical response of the cells to different mechanical microenvironments was confirmed through reduced circadian rhythms also in 3D cell cultures, when the expression of vinculin (a type of protein found at the integrins that play an important role in cellular mechanosensing) was reduced (i.e., knockdown technique), and the cytoskeleton of the cells was disrupted by treatment with latrunculin B (a toxin that prevents actin filaments from polymerising; Yang et al., 2017).

Rogers et al. (2017) used human stem cell models to study the cellular response to rhythmic mechanical stimulation (cell stretching on a flexible silicone substrate) at the frequency of 1 Hz for several 12-hour stimulation-rest cycles and demonstrated the synchronisation of some of the key clock gene activities, such as ARNTL and PER. Similarly, Vágó et al. (2022) demonstrated synchronisation of clock genes (ARNTL, CRY, and PER) through rhythmic uniaxial compression force (one hour per day for six days) on chondroprogenitor cells. The induced regulations of clock genes through rhythmic mechanical stimulations of the cells can be important and relevant in the context of stem cell and regenerative medicine research. This is due to the clock genes' possible interaction with stem cell differentiation regulatory mechanisms such as the WNT signalling pathway, cellular energy metabolism, early involvement for multi-lineage differentiation capacity (Ameneiro et al., 2020; Gallardo et al., 2020; B. Guo et al., 2012).

In sum, cellular rhythms have been shown to have an important function, and experimental data indicate that such rhythmic activities at the molecular level can be manipulated through mechanical rhythmic stimulations.

3.5 Summary

In this chapter of interdisciplinary discussions, the "dialogue" between biology, musicology, and physics helped to reveal some shortcomings and possibilities. The takeaway points include the following:

- Cells are a complex system that can sense mechanical alterations in their microenvironment.
- Due to the physical differences and limitations between audible sounds (including music) and the cells, it is not expected that sound pressure

waves (at least in the audible frequency range) can induce a meaningful mechanical interaction at the cellular level.

- Through induced fluid flow in cell culture that can generate shear stress, audible sounds (including music) can induce relevant mechanical alterations at the cellular level.
- Rhythm (a series of events that regularly varies and repeats, generating patterns in time) seems to be fundamental both in (cell) biology and music.

Chapter 4

Methodology

"I can't see a way through," said the boy.
"Can you see your next step?" [...]
"Just take that," said the horse.
– from *The Boy, the Mole, the Fox and the Horse* (2019)
by Charlie Mackesy

4.1 Introduction

In order to answer the research questions (as stated in Chapter 1) efficiently, the research methodology in this dissertation followed a multi-phase experimental approach. The project was divided into the following phases:

- Phase 1: Design, develop, and prototype the construction of a sound generation system that incorporates a controllable and measurable input (sound stimuli).
- Phase 2: Design, construct, and evaluate a controlled acoustic environment to be used for sound-based cell experiments.
- Phase 3: Employ the apparatus developed in phases 1 and 2 on the targeted cell culture and measure output (cellular response/readout) in collaboration with biology and physics colleagues.
- Phase 4: Optimise the stimulus-response for the targeted cell culture.

Accordingly, the methodology of this dissertation consisted of three essential components: 1) sound generation, 2) acoustic testing environment, and 3) biological output, as shown in Figure 4.1. Each component was designed to produce a quantifiable readout to increase the reliability and reproducibility of the

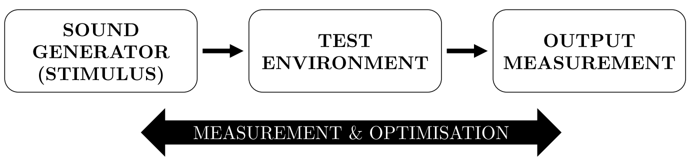

Figure 4.1: The three main components and the refining process (measurement and optimisation) of the methodology.

findings. The characterisation of the generated sounds and quantified biological output are reported in Paper IV. The design, construction, and characterisation of an acoustic testing environment are reported in Paper III. The measurement and optimisation in Figure 4.1 meant that the experimental methods (e.g., the experimental setup and biological assay) were refined through continuous characterisation and experiments.

4.1.1 Some challenges and their impact on the methodology

As could be expected, there were several hurdles when trying to perform theoretical and experimental research between three different disciplines (biology, music technology, and physics). Three aspects had a significant impact on the methodology of this dissertation.

The first challenge was my unawareness of the challenges that biologists and physicists face in their research. I did not understand their questions, and at the same time, I was struggling to formulate my own questions from my own interdisciplinary background and "home" discipline (music technology). This situation is probably true for most PhD candidates and projects, but it felt more extreme in the ABINO project's unique coalescence of research mentalities and attitudes that seemed foreign to one another. This particular situation also made it challenging to focus on my own discipline.

Secondly, closely related to the first, much time and energy had to be spent on interpreting and interposing different scientific "languages" and ways of thinking. Moving from cross- to multi- to interdisciplinary communication was a central part of my methodology.

Thirdly, due to the nature of the ABINO project, the focus was primarily on contributing to life sciences, more specifically, cell biology. For instance, developing a novel solution and/or discovering a novel understanding of the cellular process(es) were the dominant idea and at the centre of attention. In practice, this meant that reciprocal learning between disciplines was limited.

Finding solutions to overcome these three challenges helped carve my methodology more constructively. Initially, my chief research goal was only to investigate and develop different ways to manipulate audio signals through various digital signal processing techniques. As the project progressed, I felt it was important to learn more about general biology and cell biology to know

what audio signals to create and what musical elements to use and correlate with cellular processes. Additionally, to develop and characterise a biotechnology-related experimental setup, I needed a more in-depth understanding of the physical aspects of the setup (e.g., different methods of measurement and characterisation).

As a result, I was compelled to learn from my ABINO colleagues about the other disciplines. This meant that most decisions about the methodology were based on many hours and emails of discussions, which included numerous naïve questions. Furthermore, a systematic literature review resulted in Paper I, and my theoretical research into the rhythmic aspects of biological systems resulted in Paper II.

In this chapter, I will present and discuss why I decided on certain methods, and how the multi-phase approach was realised.

4.2 Phase 1: Sound generation system

4.2.1 The physical setup

My starting point was to design a controllable digital audio-based setup that could produce sounds within the audible frequency range. However, the physical limitations of using audible sounds to stimulate cell cultures directly were too great, as discussed in the previous chapter and in Paper I. For those and other practical reasons, my physics colleague from the ABINO project has been developing an ultrasound-based system that can be semi-submerged in liquid. However, since my perspective came from the field of music technology, I wanted to stay within the audible frequency range and manipulate more music-related variables: even if it meant that the effect on the cells was probably not directly from the sound pressure waves but emerging from the resonances in their environment. Suitably, an experimental apparatus suggested by the biologists from the ABINO project was a speaker-based vertical (unidirectional) vibration generator (Figure 4.2).

The apparatus was used as shown in Figure 4.2 for several preliminary experiments with an arbitrary function generator. Later, in order to manipulate the audio signal more flexibly, the system was modified so that the vibration generator was controlled by the digital audio signal that was generated from a laptop via an amplifier (Figure 4.3).

The modification also improved the portability of the setup, which was helpful since the setup had to be moved between different rooms, laboratories, and sometimes also different university buildings for experiments and measurements. Subsequently, the physical aspects of the vibration generator were measured and characterised in terms of its peak and root mean square (RMS) acceleration, displacement, and velocity using a triaxial accelerometer. Additionally, particle tracking velocimetry (PTV) was performed to test whether the liquid flows within the chamber of the cell plate, and the shear stress was estimated according to the measurement, which is reported in Paper IV in detail.

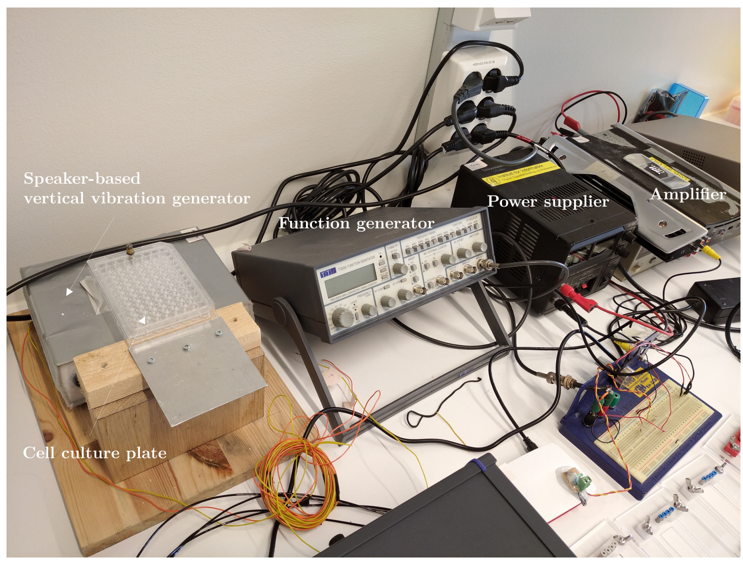

Figure 4.2: The initial speaker-based vertical vibration setup, including 3B scientific vibration generator, TG550 function generator, Manson EP-613 bench top power supplier, and JBL GTO-1004 audio amplifier.

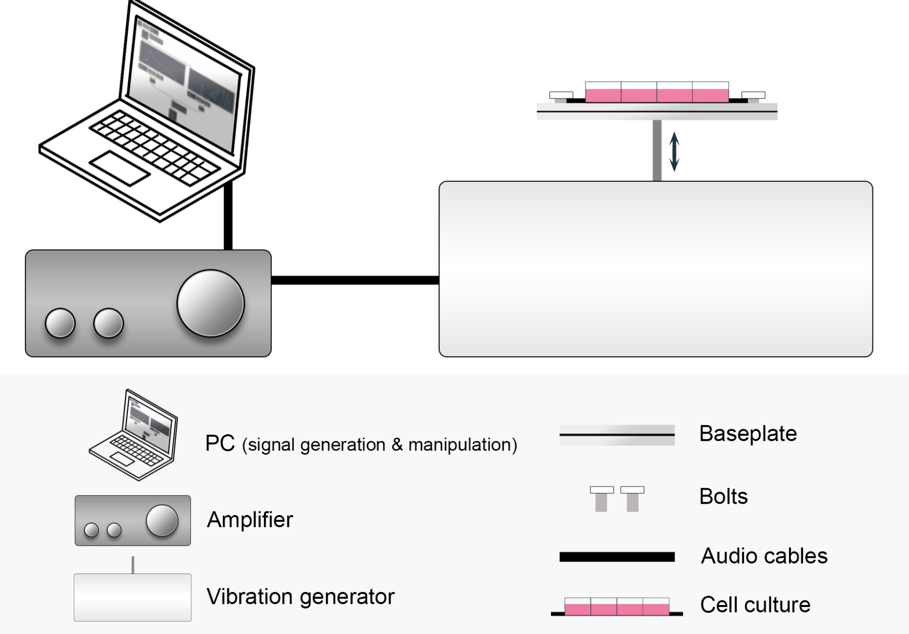

Figure 4.3: A schematic of the modified setup.

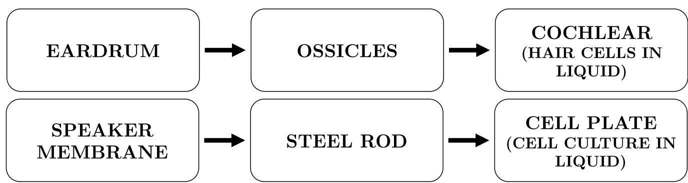

Figure 4.4: The essential parts of the experimental setup compared with the key components of the human auditory system.

At some point throughout the process, I realised the similarity between the experimental setup and the human auditory system discussed in the previous chapter. I recognised that the experimental setup models the essence of the auditory system and physiology. This is illustrated in Figure 4.4.

Although the cells that I used were non-auditory cells, one of the main aims of the ABINO project was to use sounds as cellular stimuli, and there was something to be learnt from the natural physiological system that is made to be sensitive specifically to sounds (i.e., human auditory system). The constituent parts and the basic mechanisms of the experimental setup were structurally similar to the auditory system (Figure 4.4). The mechanical movement of the eardrum is transferred via the ossicles in the middle ear to the cochlear in the inner ear. Similarly, the mechanical movement of the speaker membrane is transferred through the attached rod to the cell plate. As discussed in the previous chapter, the critical mechanism for humans to hear sound is a cellular process called mechanotransduction of the hair cells found in the liquid inside the cochlear. Thereafter, it became reasonable to think that the cell culture in liquid may sense the sound produced by the speaker through a similar systemic process. Collectively, as my main concern was creating stimuli and systems that are biologically relevant, the structural commonality with the auditory system ultimately gave me a more sensible and logical reason to stay with the setup.

4.2.2 Digital signal processing

4.2.2.1 Audio signal processing

The audio signal was generated and manipulated from the Max/MSP environment, a visual-based programming language for audio signal processing and other functions and features. I chose Max/MSP because of its extensive documentation, a large number of useful functions, so-called objects, and capability to generate a graphical user interface (GUI). The interface can be designed so it can be intuitively operated to create and control fairly sophisticated signals without extensive knowledge about the programme.

The GUI (Figure 4.5) was designed in the Max/MSP environment and exported as a standalone patch used to generate audio signals for the cell stimulation experiments.

Figure 4.5: The user interface, developed in the Max/MSP environment.

The structural design of the signal manipulation within the Max/MSP environment is illustrated in Figure 4.6.

The oscillator (generating a sine tone) was continuously producing a signal at a set frequency and amplitude. It was passed to a gate object that was used to create discrete sound "objects" at specific time intervals. Sound objects are a concept put forth by a French composer named Pierre Schaeffer (1910–1995; Schaeffer, 1967). Jensenius (2022, p. 102) describes sound objects as a discrete sound shape that can "last up to a few seconds but are often shorter" as opposed to a continuous and uninterrupted sound. In this dissertation, sound objects were controlled by various temporal factors, including pulse duration, interval, and ramp time, which resulted in a "ramp-plateau" envelope (Kim et al., 2010), which is applied to avoid digital "clicks" (Figure 4.7). This ramp function could be used to shape the signal so that the signal starts and ends more gradually.

4.2.2.2 Experimental conditions: The decisions made about the experimental variables

Initially, some conundrums were around the experimental conditions. The very first question that puzzled the ABINO team was: at which sound frequency do we stimulate the cells? To address the question, one of my early plans was to keep the amplitude the same, scan through the frequency at an arbitrary interval within the audible frequency range, and measure a corresponding biological readout. It was foreseeable that there would be some mechanical resonance frequencies in the setup I was using. Whether those resonances would be "good" or "bad" on the cells was unclear. The intention was to collect as much biological data as possible while scanning through the frequency range. This could then be

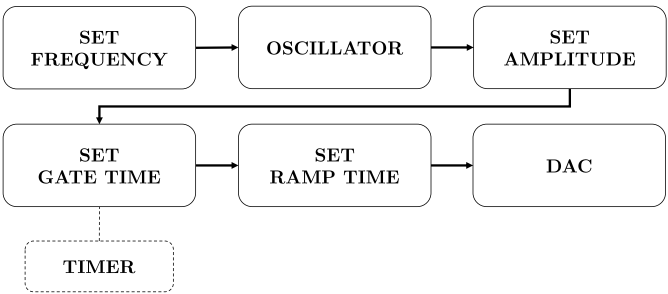

Figure 4.6: The structural design of the signal manipulation within the Max/MSP environment. An oscillator generates a continuous tone at a set frequency and amplitude. The gate regulates the signal flow at a set time, and when the gate allows the signal through, the signal is shaped ("ramped" up and down) for a set time. Eventually, the digital signal is converted into an analogue audio signal through the digital-to-analog-converter (DAC) object in the Max/MSP environment. The "homemade" timer function within the Max/MSP environment is triggered by the initial signal that passes the gate. After a set time, the timer closes the gate to prevent the signal flow.

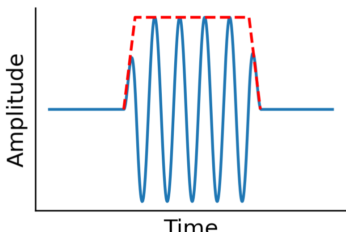

Figure 4.7: A section of an internally recorded waveform of the signal that was generated from Max/MSP. The signal is "ramped" up and down for ten milliseconds when the gate allows and prevents the signal, respectively. As a result, a ramp-plateau envelope (indicated with a red dotted line; Kim et al., 2010) is created and used to shape the signal.

used as input to a machine learning-based system that could associate frequencies with biological responses. Since collecting biological data can be an expensive process (time and budget), I hoped this approach could narrow down the most "effective" frequency or frequency range(s) in relation to the targeted cellular responses. Nevertheless, several perspectives helped me devise the plan from a different angle.

Firstly, given my musical background, I started to think that the temporal aspect of the stimulation was more important than simply the frequency. One of my RITMO colleagues, Agata Zelechowska, found that the temporal aspect of the musical stimuli (e.g., pulse, tempo, rhythmic complexity) was an important contributing factor for the listeners to move even when requested not to move in her experiment (Zelechowska, 2020, p. 102–103). Zelenchowska frames this effect of temporal variations of musical sounds on the human body movement as "irresistible" (Zelechowska, 2020, p. 106). Also, it is well established in neuroscience that rhythm activates the motion regions of the brain even when there is physical motion is executed (Chen et al., 2008). In addition to these findings, the likeliness of the *indirect* effect of audible sound waves on the cells shifted my focus away from sound frequency. My contemplation of these ideas led me to the second point.

Secondly, I was mostly concerned with how to stimulate the cells in a physiologically and biologically relevant way? This is rather an exploratory question. What is relevant can vary greatly depending on different cell models. For example, mimicking the *in vivo* tissue environment using various biomaterials has been investigated for many decades and seen encouraging progress due to several technical developments that can produce bioscaffolds using bioink and 3D bioprinting (G. Huang et al., 2019; Rijal and Li, 2018). However, I was looking for something related to temporal variations that can be broadly applicable. The area that seems to remain under the radar in life sciences research is how often and dynamically the cells are probed with different types of mechanical stimuli. As I laid out in the previous chapter, rhythm is an essential element both in cell biology and music. This was an inspiring concept, and I decided to design stimuli that are rhythmical, defined as a series of events that regularly varies and repeats, generating patterns in time in Chapter 3.

Thirdly, one of the main aims of the ABINO project was to develop a novel hiPSCs differentiation protocol to generate insulin-producing cells in the laboratory. Related to this aim and the second point above, I thought about where insulin-producing cells are naturally found in the body. The insulin-producing cells named β-cells are found only in the pancreas, more specifically in pancreatic islets (Bonner-Weir and Weir, 2009). The pancreas is known to be vascularised from the early stage (i.e., embryogenesis; Covantev et al., 2017; Duvillie, 2013), and the pancreatic islets in the pancreas are highly vascularised (Jansson et al., 2016; Muratore et al., 2021). β-cells are located in these islets and coupled to these blood vessels for functional reasons, for example, to rapidly sense the fluctuations in glucose level in the bloodstream (Jansson et al., 2016). This was probably a naïve perspective, but I thought about the fact that the blood flow rate is regulated by the rhythmicity of the cardiovascular system:

the constriction and dilation of the smooth muscle cells in the vessel walls and the pumping of the heart. Furthermore, β-cells "reach out" to be physically coupled to blood vessels in the pancreas. Accordingly, I hypothesised that β-cells are found in a microenvironment that is biochemically and physically rhythmic. Although I used HeLa cells (a cervical cancer-derived cell line) in the experiments, these were relevant facts that could be used as a foundation for later experiments that may involve hiPSCs differentiation protocol towards generating insulin-producing cells.

Lastly, when I considered the heart rhythm, I noticed the importance of regularity between each heartbeat. Although it seems regular, at the microsecond level, the heartbeats vary constantly. This dynamic and rhythmic temporal aspect of the heart has critical health implications. For instance, reduced heart rate variability is used as a marker of higher risks for cardiovascular-related diseases (Assoumou et al., 2010; Lombardi et al., 2001; Nantsupawat et al., 2022). In some cases, irregular heartbeats or a skipping of a heartbeat (arrhythmia) can also result in serious diseases (Antzelevitch and Burashnikov, 2011). Therefore, maintaining a certain level of variability is paramount for the cardiovascular system to function resiliently. From this, the follow-up hypothesis was that slightly varied mechanical cues could be biologically more relevant than ones that are continuous (uninterrupted) or rigidly regular.

To keep things simple, the experimental parameters were kept to three basic signal processing features: sound frequency, amplitude, and waveform. Although the waveform was a static parameter (most studies used only sine tones), these three parameters were the most common that featured in the literature as indicated in Paper I. Additionally, a temporal regulation of the digital signal flow using a gate function in the signal chain was employed to generate and control the rhythmic experimental variables.

The regulation of the flow of the continuous signal from the oscillator followed three simple principles to create three different experimental conditions:

1. Continuous Vibration (CV): A continuous (uninterrupted) signal by bypassing the gate
2. Regular Pulse (RP): Metronomically regular pulses by setting the gate opening and closing time the same throughout the experiment
3. Variable Pulse (VP): Irregular pulses by setting the gate time that varied pseudorandomly between set upper and lower thresholds

Additionally, No Vibration (NV) was used as a control condition.

4.2.2.3 Designing and generating the rhythmic conditions: Generation of a pseudorandom number sequence

For the RP condition, the gate time was fixed in the experiments performed in Paper IV. This meant that the pulse duration and interval between consecutive pulses were kept numerically the same. Accordingly, the condition was named "Regular Pulse".

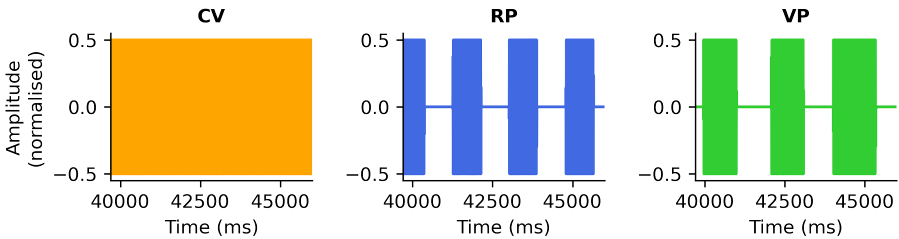

Figure 4.8: A section of the internally recorded audio signal of different experimental conditions compared. CV: Continuous Vibration. RP: Regular Pulse. VP: Variable Pulse.

For the VP condition, the number sequences were created using the *random* object in the Max/MSP environment. However, these "random" numbers are not truly random, as Wakefield and Taylor (2022, p. 91) states:

> These aren't truly random numbers but are an algorithmically generated sequence of values that are evenly distributed, which means any value is roughly as likely to appear as any other. The sequence is so complex as to have no discernible pattern, making it practically quite unpredictable.

Therefore, the number sequences were only pseudorandom. The sequence was also not chaotic. Chaos, as Wakefield and Taylor (2022, p. 112) defines, is:

> [...] a class of mathematical equations whose output over time is also quite deterministic but nevertheless unpredictable in a way that is different from the deterministic unpredictability of noise. [...] It might hold steady for a while in one such regime before suddenly switching to another. Or, more simply, it might seem to oscillate in a characteristic way but which nevertheless, unlike a typical periodic LFO, does not exactly ever repeat.

The "random" number sequence generation was controlled in the sense that the lower and the upper limits of the number generations could be limited. For instance, only the numbers between 1,000 and 1,500 were allowed to be generated "randomly" in the experiments. This condition was named "Variable Pulse".

To illustrate these differences between the three conditions, the audio signals were recorded and shown in Figure 4.8.

The reason for the two different rhythmic variables was that I wanted to study how the cells would respond to different levels of temporal regularity: highly ordered patterns versus unpredictably fluctuating patterns.

4.3 Phase 2: Controllable acoustic experimentation environment

In an effort to create a controllable acoustic environment, a mini-acoustic chamber was designed and constructed. The construction and characterisation of the chamber are reported in-depth in Paper III. In general, acoustic-related experiments that need accurate sound (re)production and/or measurements are conducted in an acoustically treated room, typically an anechoic chamber designed to completely absorb sound reflections. However, access to such space is scarce and can be costly. Additionally, biological cells can be sensitive to many environmental factors, for instance, through biomaterials, biochemicals, mechanical forces, and the timing of these stimulations (Goetzke et al., 2018). For these reasons, a more easily accessible and controlled experimental space was necessary, and an economic mini-acoustic chamber was constructed as shown in Figure 4.9.

4.4 Phase 3: Biological cells and assays

4.4.1 Cell cultures: Selection of cells and the maintenance

From the early stage of my PhD studies, I was trained to do some basic cell work in the biology laboratories at the Hybrid Technology Hub (HTH) facility situated at the Faculty of Medicine, University of Oslo. Although basic, doing the actual hands-on cell works in the lab helped me to better understand biologists' research problems. After several months, I could manage to grow and maintain cells mostly on my own and prepare them for the experiments.

One of the ABINO project's ultimate goals was to develop or enhance existing differentiation protocols for human induced pluripotent stem cells (hiPSCs) to generate insulin-producing cells in the laboratory for the purpose of treating diabetic patients. The protocol to generate hiPSCs was developed by Japanese researchers in 2007 (Takahashi et al., 2007); they first reported on iPSCs generation using mouse fibroblast cells in 2006 (Takahashi and Yamanaka, 2006). These works were groundbreaking advancements in biology and medical research since iPSCs are reprogrammed embryo-like stem cells from an adult cell, such as skin cells, that can potentially be developed into many other cell types in the body (C.-Y. Huang et al., 2019). Furthermore, they function similarly to embryonic stem cells (ESCs) and are not subject to ethical issues of ESCs.

However, such primary cell lines—for example, cells isolated directly from human tissue—can be expensive (both in time and budget) to attain and maintain. It has been reported that it can cost up to $800,000 and 6–9 months to generate clinically proven iPSCs, although the cost of the cell lines for research purposes can be less (C.-Y. Huang et al., 2019). There are efforts to reduce the cost and time to generate and maintain hiPSCs (Liu et al., 2020; Lyra-Leite et al., 2021; Okumura et al., 2019), but it is still not an "easy" cell line to work with. These cell lines would typically be purchased from other institutions for research. Taking these economical factors into account, it was a better strategy to use a

Figure 4.9: A fully constructed mini acoustic chamber. (Top left) Side view of the bottom part of the top cover. (Top right) Top view of the top cover. (Bottom left) Inside the chamber. (Bottom right) Fully assembled chamber. The two handles are used to lift open the top cover. The round hole in the centre is for accessing the chamber for measurement (e.g., microphone) and observation (e.g., digital microscope).

cell line that is cheaper and easy to work with to prototype an experimental setup and protocol for the purpose of this dissertation.

For these reasons, another human cell line, so-called HeLa cells, was used for the experiments. HeLa cells originated from a cervical cancer tissue of a woman named Henrietta Lacks, hence the name, at the John Hopkins Hospital in Baltimore, the United States (Landry et al., 2013). HeLa cells were the first established human cell line in a laboratory since its isolation from the patient in the early 1950s and have led to many important discoveries in biology and medicine research fields (Landry et al., 2013). One noteworthy work was by Harald zur Hausen, who discovered the correlation between the human papillomavirus and cervical cancer by studying HeLa cells (Durst et al., 1983). Later, the discovery helped to develop a vaccine against the virus, and Harald zur Hausen won a Nobel Prize[1] in Physiology or Medicine in 2008.

After many decades of its use in laboratories around the world, the cell line's reliability has been questioned due to its abnormal characteristics (Landry et al., 2013). However, HeLa cells are still in use by many biology laboratories and research groups and are a useful model of human biology due to their cost-effectiveness. At the HTH, HeLa cells were already in culture, and I could easily access the cell line.

In vitro cells can be cultured in various ways. A typical method is to grow the cells in a flask with cell media (liquid with biochemical factors for maintaining and growing cells). Generally, the cells would attach to the surface of the flask and be submerged in the cell media. As the cells grow and divide, the flask can be overpopulated. Then, it is necessary to "passage" the cells, meaning to detach the cells from the flask using a certain type of enzyme (e.g., trypsin) and discard a portion of the cells. The remaining cells would be "re-seeded" in the flask with fresh cell media. That could be passage number one, and the next round would be passage number two and so forth. The cell line that I used was initially frozen, so they were thawed and passaged a few times to let the cells stabilise before the experiments. For the experiments, cells were detached from the flask and moved to a particular type of cell chamber slides (Figure 4.10). This specific type of chamber slide was more suitable than a common Petri dish for the high-resolution inverted microscope with the oil immersion technique that was available at the facility.

4.4.2 Biological assays: Where do I look in the cells?

There are many types of biological procedures (assays) one can perform, and the type of experimental methods for assays depends on what needs to be analysed in the cells. Determining what part of the cells to study was unclear at the beginning of my PhD project. What helped, in the end, was understanding how cells would sense their environment, as discussed in the previous chapter.

One of the important parts of the mechanical sensing mechanisms of the cells is the cytoskeleton. The cytoskeleton consists of three main components:

[1] https://www.nobelprize.org/prizes/medicine/2008/hausen/facts/

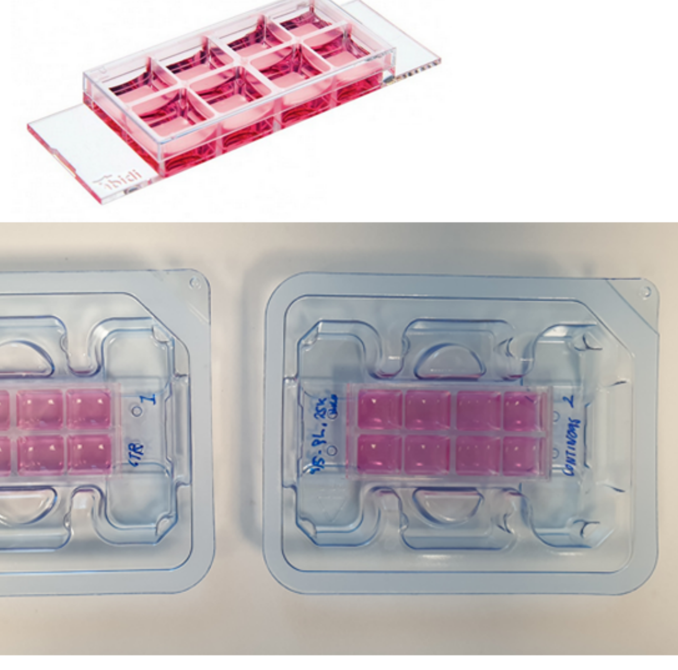

Figure 4.10: (Top) ibidi μ-slide 8-well glass bottom coverslip. Image: ibidi. (Middle) Cells were moved from a flask to the ibidi slides for the experiments. (Bottom) A diagram showing the bottom thickness of the ibidi slide from a side view. The chamber slide was purchased from ibidi. The thin glass bottom enables to image the cells using an inverted microscope with the oil immersion technique.

microtubules, actin filaments, and intermediate filaments (O'Connor and Adams, 2010). In particular, actin filaments form the major part of the cytoskeleton and can be found in bundles as the cortex (near or under the cell membrane), as stress fibers (more in the "body" of the cells, but outside the nucleus), or as lamellipodia or filopodia (at the "front" end of the cells as protrusions when cells are migrating) (Cooper, 2000). Actin filaments are helical structures composed mainly of actin proteins arranged to form a string: the actin monomers knowns as globular actins, or simply G-actins, can polymerise and form filamentous actins called F-actin filaments. The actin filaments' general function is to provide shape and strength to the cells and be actively involved in cell migration and division processes (Cooper, 2000).

Actin filaments are a dynamic structure, and their polymerisation and depolymerisation could happen in a matter of tens of seconds (Priya and Yap, 2015). It has been shown previously that mechanotransduction mediated through actin filaments can be as fast as 300 milliseconds and up to 40 times faster than biochemical stimulation (Na et al., 2008). This fast-response aspect was appealing since one could study the mechanical responses of the cells relatively quickly. Another consideration was that the method to check these structural changes was not extremely costly. The filaments' structural changes (e.g., their length, thickness, and angle) can be used as a helpful indicator that the cells are responding to the stimuli in a certain way, even though what the changes imply can be difficult to interpret and needs to be investigated further.

On top of the economic advantage, actin filaments form a part of the key mechanisms of how mechanical cues are transferred into the cells and lead to biochemical or gene expression alternations (i.e., mechanotransduction; Wang et al., 2009). Actin filaments, particularly stress fibers, are attached to focal adhesions, where integrins (proteins found in the cell membrane and responsible for mechanosensing; Wang, 2017) can bind to the ECM (extracellular matrix, essentially micro extracellular environment with different types of proteins for the cells to bind to; Totsukawa et al., 2000).

A potential mechanism of actin filaments acting as a bridge between focal or surface adhesions of the cells and the cytoplasm and nucleus has been alluded to by Wang et al. (2009). This is because actin filaments can also be connected to the nuclear scaffolding around the nucleus, indicating mechanical stimulations could have a long-range impact on the nucleus (Maniotis et al., 1997). Moreover, the actin cytoskeleton can influence cell signalling pathways by various additional biochemical mechanisms, including sequestering (i.e., holding back) and releasing cell signalling regulators (Moujaber and Stochaj, 2020). This meant that the experiment might lead to further investigations of the underlying mechanisms or effects of mechanotransduction involving actin filaments. This mechanism is currently not entirely understood, and investigating structural properties and/or responses to mechanical stimuli of actin filaments appeared to be an area that could make a meaningful contribution to the field.

Taking the above into consideration, my biology colleague from the ABINO project and I decided to investigate and focus on the structural properties and changes of the F-actin filaments of the cells under different mechanical stimulation

conditions and compare the possible differences between the conditions.

4.4.3 Data collection: Fluorescence microscopic images

The main methodology employed for data collection was through fluorescence microscopic images. Prior to putting the cells under a microscope, the cell had to be prepared in a specific way. The standard protocol I had to follow in sequence after the stimulation was the following:

1. Fixation of the cells
2. Permeabilisation of the cells
3. Fluorescent staining of the F-actin filaments in the cells

When cells are fixed, cells and their components are inactivated and immobilised (B. Q. Huang and Yeung, 2015). We achieved this by using a paraformaldehyde (PFA) fixation agent after the stimulation. As a brief example, the protocol I followed to fix the cells followed the steps below:

1. Wash the cells with phosphate-buffered saline (PBS)
2. Add 0.5 ml of 4 % PFA per chamber
3. Leave it at room temperature for 15 minutes
4. Wash the cells with PBS twice
5. Store the fixed cells in a fridge at +4°C

After the cells were fixed, stable pores in the cell membranes needed to be created to access the cells with fluorescent staining agents. Usually, a type of detergent, such as Triton X-100, is used to permeabilise (making holes in the membrane or removing the lipid membrane) the cells (Koley and Bard, 2010).

Following the fixation and permeabilisation of the cells, Alexa Fluor™ 488 Phalloidin[2] was used to stain the F-actin filaments in the cells. The two main "ingredients" of the agent are phalloidin and a fluorescent dye.

Phalloidin is a type of toxin found in a specific type of mushroom called *Amanita phalloides* (Adams and Pringle, 1991); known to cause serious liver damage (Singh and Sharma, 2022). Phalloidin can selectively bind to the actins in the cells (Adams and Pringle, 1991). Utilising this unique property, phalloidin is used to conjugate with a fluorescent dye, making it possible to selectively visualise the F-actin filaments under a fluorescence microscope. The Alexa Fluor™ 488 Phalloidin, for example, consists of phalloidin conjugated to a green fluorescent dye. There is also green fluorescent protein (GFP), which was discovered in a jellyfish known as *Aequorea victoria* by Shimomura et al. (1962). The GFP has been widely used in laboratories to study the expression of genes and proteins in cells (Tsien, 1998), but this is different from a fluorescent dye.

[2]https://www.thermofisher.com/order/catalog/product/A12379

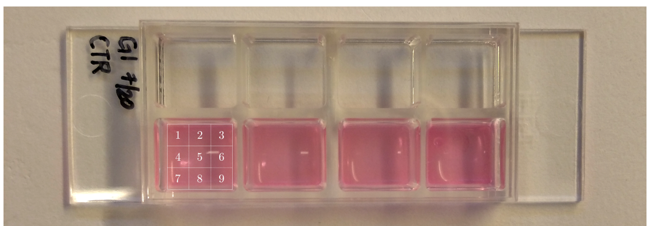

Figure 4.11: An illustration of how a chamber was “divided” when images were taken under the microscope.

Generally, a fluorescent dye is used in fixed cells, whereas fluorescent protein can be used as reporters in living cells.

Fluorescent dye can be excited at a certain excitation spectrum, a range of fluorescent light wavelength in nanometer (nm), and emit light at a certain emission spectrum, which will be lower in energy and a longer wavelength range than the excitation spectrum (Lakowicz, 2006, p. 5). The Alexa Fluor™ 488 Phalloidin is reported to have an excitation peak at 488 nm, hence the name, and an emission peak at 496 nm.[3]

After a couple of preliminary experiments, several fluorescence microscopic images were acquired per chamber. In order to get a more overall status of the cells and minimise the sample bias (Quinn, 2002, p. 14), an arbitrary location in each “divided” section was imaged (Figure 4.11). Accordingly, in total, nine images were taken per chamber.

Prior to imaging, the nuclei of the cells were also stained with 4’,6-diamidino-2-phenylindole (DAPI). Similar concept to phalloidin and Alexa488, the DAPI staining is added into the cells and helps to visualise the nuclei (DAPI binds to DNA) of the cells and to count the number of cells within the view field as shown in Figure 4.12.

The field of view was relatively large (40x magnification objective), and it helped to capture many cells per image. However, that meant that it was difficult to visualise the F-actin filaments accurately and, as a consequence, made the feature extraction from the images more difficult. Later, we decided to image the same experiment again but under a different fluorescence microscope with a higher resolution capability and an objective with higher magnification power (63x), resulting in a smaller field of view. For that reason, as it is reported in Paper IV, a ZEISS Elyra PS1 microscope system with standard filter sets and laser lines with an oil immersion objective (Plan-APOCHROMAT, 63x/1,4 NA Oil Ph3) was used.

We also employed a maximum intensity projection (MIP) method to improve the visibility of the F-actin filaments in the cells. MIP was originally called the maximum activity projection algorithm and was invented by Jerold W. Wallis

[3]Information retrieved from https://www.thermofisher.com and https://app.fluorofinder.com.

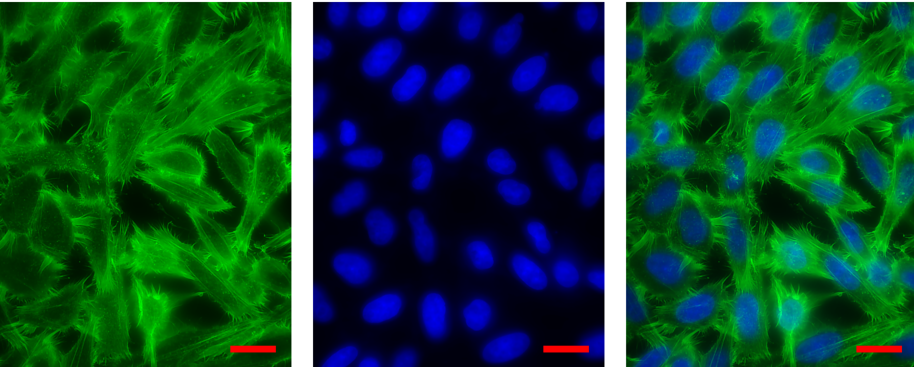

Figure 4.12: Single plane fluorescence microscopic images. (Left) A visual representation of F-actin filaments of HeLa cells stained with phalloidin green-fluorescent Alexa 488 dye. (Middle) A visual representation of the nuclei of HeLa cells stained with DAPI. (Right) The left and middle images merged. Scale bars are 20 μm. The images were cropped.

in 1989 (Wallis et al., 1989). This is a visualisation technique that can take 3D image data, such as z-stack images, into a 2D image by taking the brightest pixel in each layer. To perform MIP, z-stack images were taken (Figure 4.13) to capture and visualise a more accurate status of the F-actin filaments in the cells. The height of the HeLa cells can be up to $\sim$ 4 μm at the centre of the cell when cultured in the 2D system on a hard flat surface (Guan et al., 2017). In our experiments, we found that the range of the stack could be kept at 1.54 μm using 15 planes and still capture an overall 3D representation of the F-actin filaments of the cells. Thus, the interval between each z-stack plane was $\sim$ 0.11 μm.

Consequently, I was able to acquire images with a smaller view field, capturing less number of cells, but a more defined structural representation of the F-actin filaments in the cells. An example of the resulting images is shown in Figure 4.14.

Eventually, I acquired 108 microscopic images representing F-actin filaments of HeLa cells from the series of experiments I performed.

To explain briefly how the number came about, I had three experimental variables and one control. In total, I had four conditions (NV, CV, RP, and VP). As shown in Figure 4.11, a total of nine images were taken per condition. Therefore, I ended up with 36 images for one experiment. In addition, the experiments were repeated three times after the initial experiment to obtain reliable data. The experimental conditions were kept exactly the same, and the same type of cells (HeLa) were used. However, in each experiment, the cells were from a new batch that was thawed and stabilised in the laboratory (biological replicates: “the same type of organism treated or grown in the same conditions”; Bell, 2016). Unfortunately, in one of the experiments, the F-actin filament staining protocol did not work so well and was not included in the final analysis. In the end, I had a total of three experiments with quality image data.

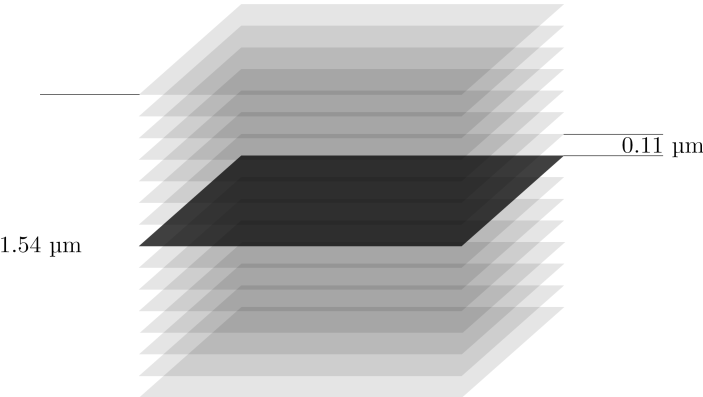

Figure 4.13: An illustration of the z-stack planes used to take maximum intensity projection images. The stack images were taken above and below the “centre” (black) plane.

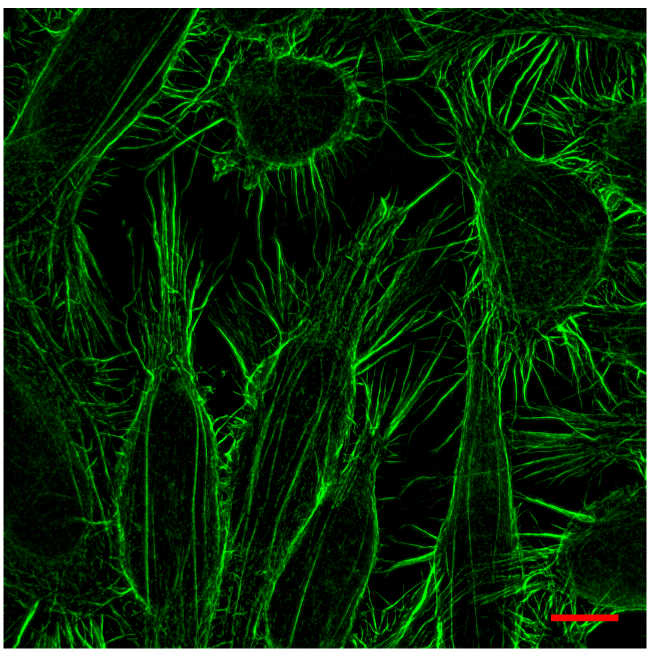

Figure 4.14: MIP visualisation of F-actin filaments of HeLa cells in a smaller view field with a stronger magnification objective. The scale bar is 20 μm.

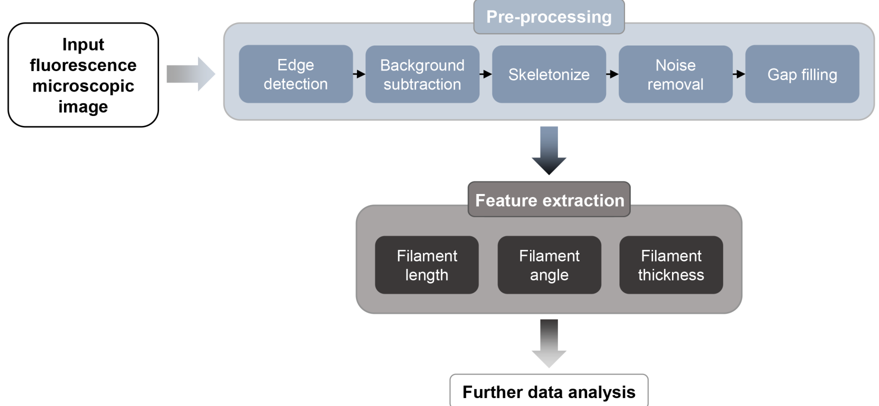

Figure 4.15: A schematic of the steps taken in the algorithm to extract features from the microscopic images.

4.4.4 Data analysis: Microscopic image analysis

After a qualitative examination of the images, it was necessary to perform a quantitative feature extraction to analyse the data further. No existing software was specifically designed to analyse the structural properties of F-actin filaments, and I had no prior knowledge or experience in image analysis. ImageJ,[4] a Java-based image processing program, is often used to analyse microscopic images in biology research. However, there was no standard plug-in for the required purpose, so I had to create my own protocol to process the images to produce the data I needed.

With a fairly steep learning curve, I managed to develop a computational algorithm with the help of my physics colleague in the ABINO project. This was implemented as a Python script, mainly using Scikit and Scipy Python image packages to automate the image processing and feature extraction. Python was utilised because of the fact that it is an open-source and high-level programming language and has been widely used for various purposes, including numeric computation for science, web development, and other software developments (Miura and Sladoje, 2022). Due to its popular demand, active community, and a great number of libraries and documentation, Python has been used frequently by beginners in programming but also for advanced bioimaging analysis (Miura and Sladoje, 2022). Scikit is a well-documented image processing library[5] and contains advanced computer vision functions, such as edge detection, peak local maxima, and other various filters (Walt et al., 2014).

The algorithm followed specific steps to process the microscopic images as shown in Figure 4.15 and with a microscopic sample image in Figure 4.16.

[4]https://imagej.net
[5]https://scikit-image.org

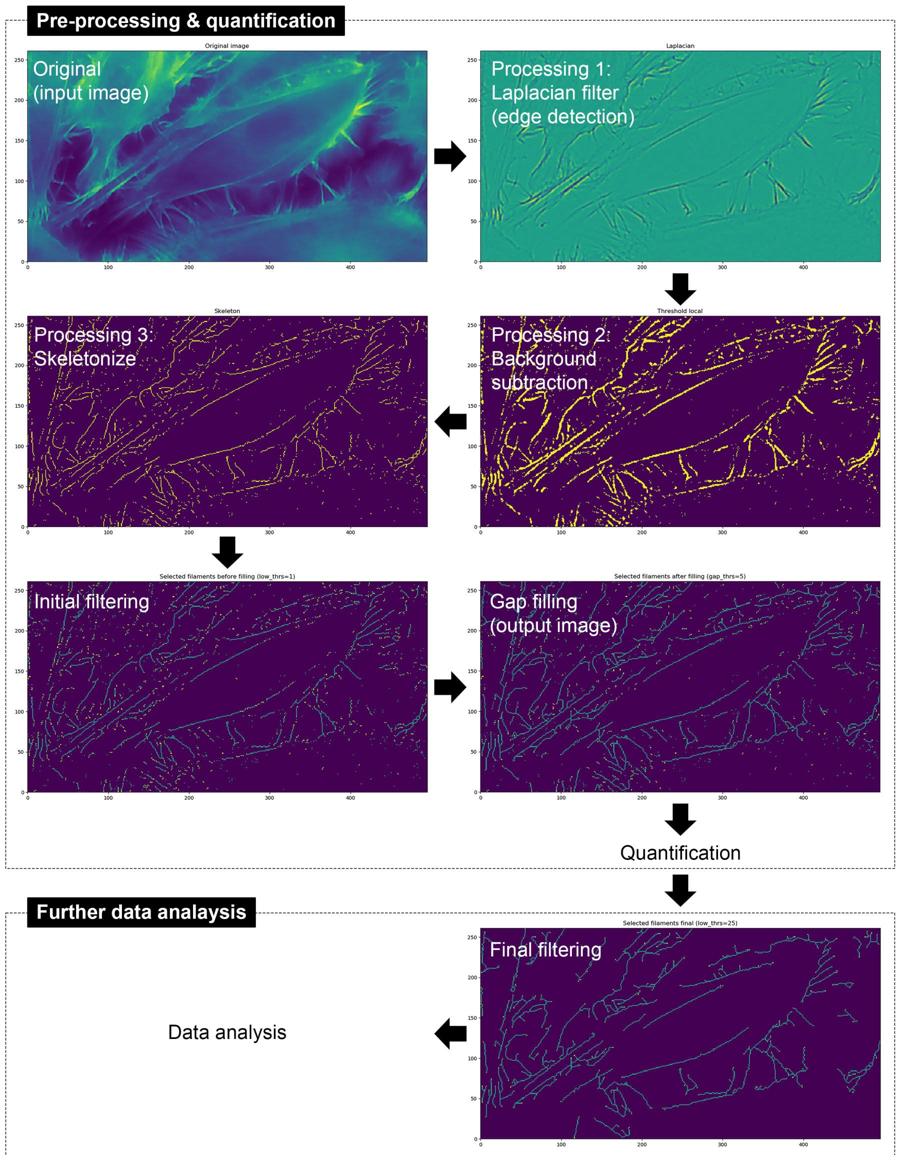

Figure 4.16: An illustration of the results of the image processing performed by the algorithm. Note that the image representation of the "Final filtering" is done only during the post-analysis of the quantified image data to filter the data points if necessary.

The algorithm was characterised and tested for its robustness. I used randomly selected samples from the collected image data from the experiment for the tests.

4.4.4.1 Test 1: The effect of blurring of microscopic images on filament length, thickness, and angle

As blurriness may occur in microscopic images, a small section of the collected image was used to test how different blur strengths can affect the feature extraction process (Figure 4.17). Subsequently, I analysed the results further in a Jupyter Notebook, an open-source web-based computing environment able to run Python code.

The blur test for filament lengths and thicknesses revealed that the algorithm produced stable results over the first three levels of the blur strength, which most probably falls within the range of blurriness that could occur in the collected microscopic images. The differences seen with higher sigma values can be considered extreme, but the results were predictable.

The angle of the filaments was similarly steady until sigma 4 as illustrated in Figure 4.18. In the figure, radians were used to plot the histograms. Generally, 0 to 360 degrees are the same as 0 rad to 2π rad. However, the data represents merely angles and not directions. Accordingly, the range is between -90 and 90 degrees; hence, -$\pi/2$ and $\pi/2$ are used for the circular histogram.

4.4.4.2 Test 2: The effect of the algorithm parameters on filament length, thickness, and angle

We have set a few parameters in the algorithm that can be controlled as necessary. The parameters included what we refer to as initial filtering (IF), gap-filling (GF), and final filtering (FF). IF defines the minimum length of the filament to be included during the image processing and quantification stage. GF defines how far the algorithm will search for filaments that are possibly "lost connections" in the neighbouring pixels. To describe this function briefly, GF was developed to compensate for the "broken" filaments in the output images where the pixel representations of F-actin filaments are sometimes disconnected. For instance, a long filament in the input image would be represented as two or three broken pieces of filaments in the output image and creates a gap(s). The function will search a potentially lost filament(s) in the neighbouring area defined by the GF value. Then, it will select a filament that matches the angle of the first filament where the search started. FF was employed after the image quantification was done to filter out data points we considered as noise.

The strategy was to find reasonable parameters to obtain the most useful and reliable data. For example, low IF will cause the algorithm to take extremely long or never finish the feature extraction. Likewise, setting an inappropriately high GF would cause the algorithm to connect all the pixels together, which is undesirable. Therefore, I took a sample from one of the collected microscopic images, tested various parameter values, and compared the results (Figure 4.19).

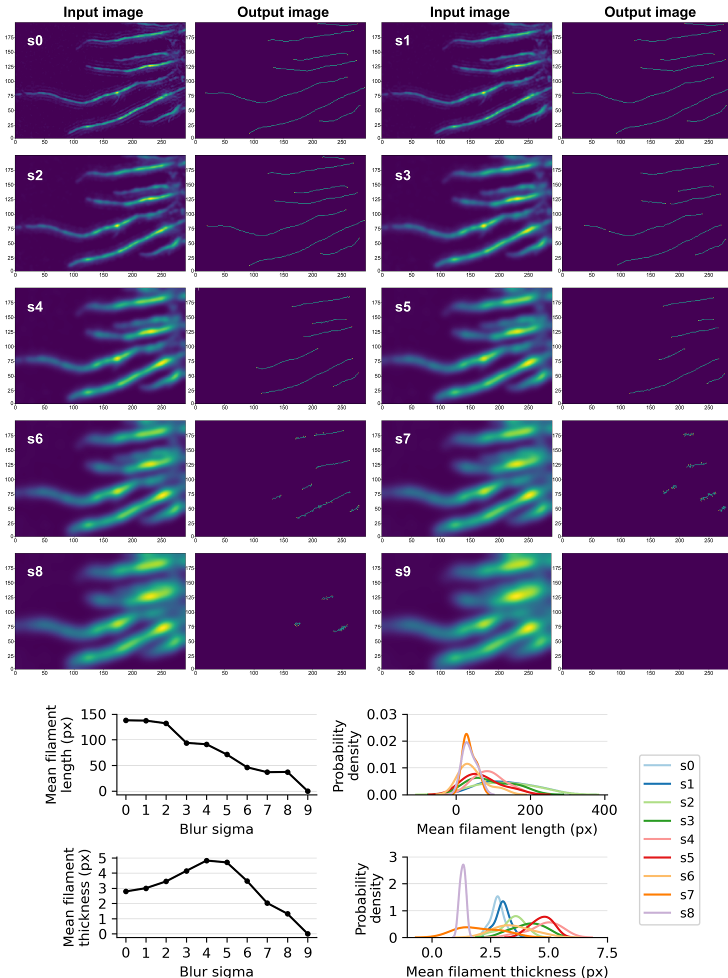

Figure 4.17: The results of the Gaussian blur test using different sigma (value determining the amount of blur) values in terms of filament lengths and thicknesses. The sigma values ranged from 0 to 9 (indicated as s0 to s9). S0 was the original image. S9 was the highest limit before the algorithm could not recognise any filament. As the sigma was increased, the number of pixel representations of the filaments reduced in the output images. Accordingly, the result of the mean filament length was stable up to s4 and started decreasing subsequently. The distribution stayed normal throughout the experiment. However, the width of the distribution narrowed because fewer filaments were identified, and shorter filaments were "lost" earlier than longer filaments from the original image. The mean thickness increased towards the middle of the sigma range but decreased after s6. One pixel represents ~ 0.148 μm in these images.

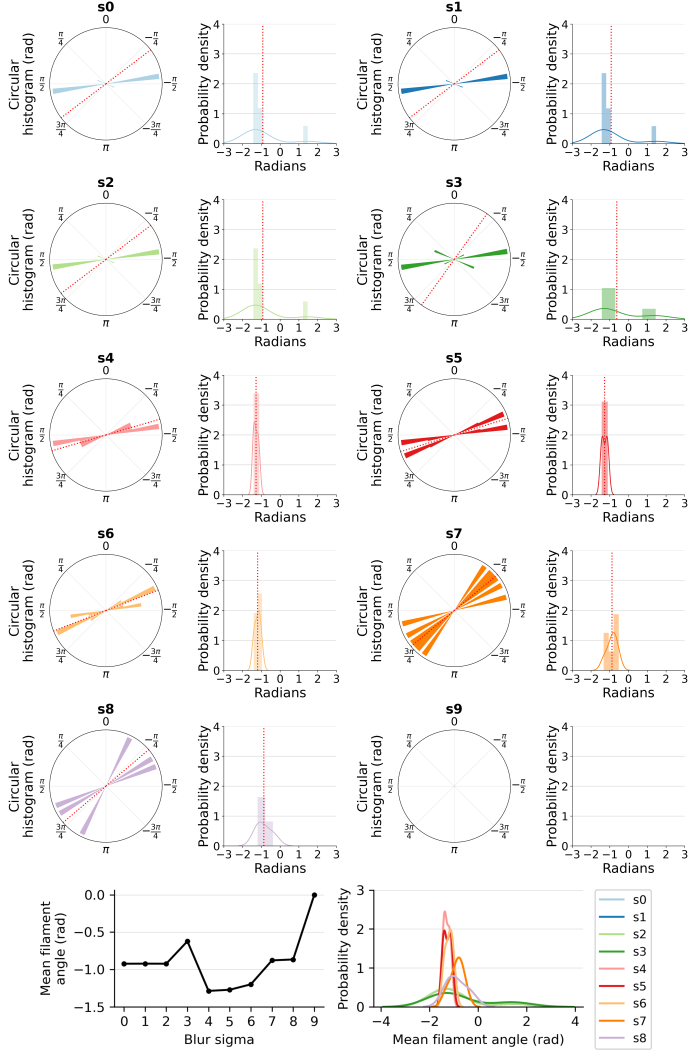

Figure 4.18: The results of the blur test in terms of filament angles, not directions. The circular histogram (the matplotlib polar plot in Python) in radians helps to visualise the angles of the filament representations in the output images in Figure 4.17. The red-dotted lines indicate the mean in each result. The angle distribution shows the relative amount of filaments at a specific angle. As was the case with filament lengths and thicknesses, a decrease in the number of identified filaments from s4 affected the results negatively. Thus, similar to results shown in Figure 4.17, the width of the distribution becomes narrower from s4. However, the mean angle of the filaments altered more unpredictably than the mean length and thickness, although it remained generally steady until s3. As indicated in Figure 4.17, no filaments were identified in s9; therefore, no data was produced for that blur strength.

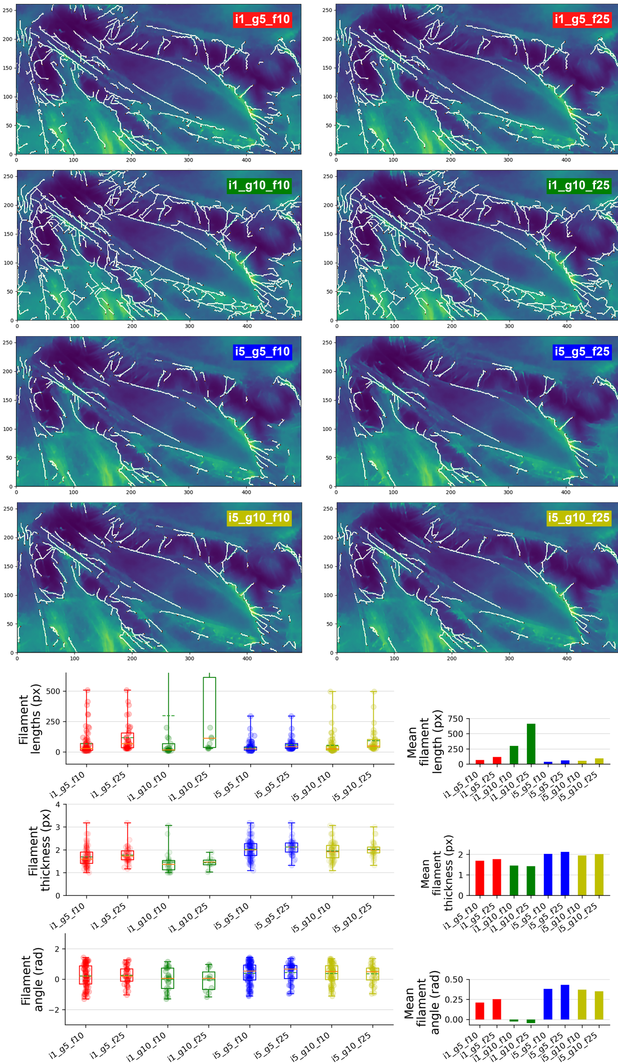

Figure 4.19: Superimposed pixel representations of the F-actin filaments on the original microscopic image and the results of the parameter test in terms of filament length, thickness, and angle. The box plots show the distribution of the data. The box represents the interquartile (the middle 50%) of the data points, the green dotted lines represent the mean, and the orange lines represent the median. The bar plots show the mean values of each parameter condition. The different colour groups include a set of parameters where only one out of three was varied. For example, in the red group, IF and GF were kept the same while the FF was varied from 10 to 25.

It became clear that certain combinations of parameters produced extremely artificial outcomes, for example, the green group (Figure 4.19). One of the contributing factors can be the low IT (red and green groups). A low IT could include more extremely short adjoining pixels or a pixel, but it was prone to more errors. Increasing the GF affected the filament lengths but did not significantly affect the filament thickness (e.g., comparing the blue and yellow groups). Finally, increasing the FF generally increased the mean value since it filtered out more short filaments.

All things considered, a set of parameters used in the blue group produced the most reliable and reasonable outcome. Since F-actin filaments can generally be short except in long-stress fibres, the lower FF was more appropriate, which could include data points of shorter filament lengths. Therefore, I could determine that the IF value of 5, GF value of 5, and FF value of 10 yielded the best outcome. Accordingly, all the data analyses employed this specific set of parameters.

4.4.5 Statistics

4.4.5.1 Statistics on microscopic image data while considering basic cell biology

After the feature extraction of the microscopic images in terms of filament length, thickness, and angle, I imported and further analysed the data in a Jupyter Notebook. In Paper IV, the employed statistics aimed to discern any significant differences between experimental conditions. The hypotheses were:

- Null hypothesis (H_0): CV (Continuous Vibration), RP (Regular Pulse), and VP (Variable Pulse) conditions do not alter the F-actin filament structures (length, thickness, angle) compared to NV (No Vibration, control).
- Alternative hypothesis (H_a): CV, RP, and VP conditions do alter the F-actin filament structures compared to NV.

There were sub-questions for the statistical tests:

- Do CV, RP, and VP have the same effect on the F-actin filament structures?
- Do RP and VP have the same effect on the F-actin filament structures?

The data sets had skewed distributions; positively skewed, as illustrated in Figure 4.20. The data points in the extreme tail could have been considered outliers. However, the data sets could display a great number of short filaments and a few longer filaments. Since the mechanical stimulation was performed globally over the whole cell plate, the microscopic images I acquired were to capture the general “impression” (inhomogeneous mixture) of the F-actin filaments in the cell culture where there are many cells and not a single cell. Therefore, it is possible that the images captured the cells at various cellular states or processes, in which F-actin filament structures would be different. Consequently, it is possible that the outliers could, in fact, be important.

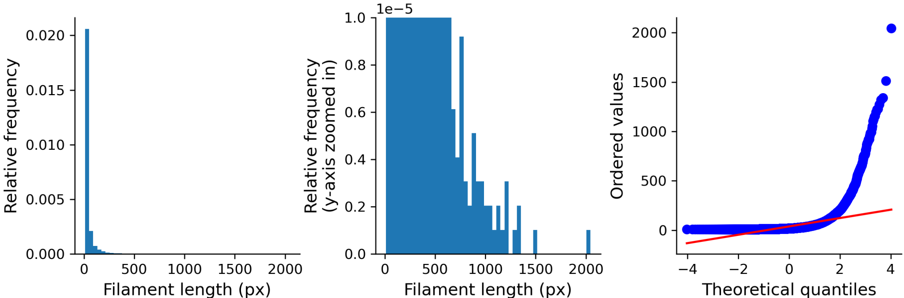

Figure 4.20: (Left) An illustration of the data distribution of F-actin filament length using a histogram. (Middle) The distribution is positively skewed with a few extreme outliers, as shown in the histogram with the zoomed-in y-axis. (Right) The Quantile-Quantile (Q-Q) probability plot was used to compare the quantified data (y-axis) and theoretical data (x-axis). The deviation from the red line suggests that the quantified data shows a non-linear pattern and is not normally distributed.

Yet, there were some extreme outliers filtered out in the PF stage since it was qualitatively determined that it was not possible for a filament to be "extraordinarily" long within the employed view field.

To compare the data sets that are largely skewed with similar variance, rank-based (non-parametric) statistical tests were employed, namely the Kruskal-Wallis, and Wilcoxon Rank sums as post hoc tests (Quinn, 2002, p. 195–196). These tests compared the medians of the data sets, as it is "a better estimator of the center of skewed distributions and is more resistant to outliers" (Quinn, 2002, p. 15). The H_0 was tested for that "there is no difference in the location of the distributions between groups or treatments and is based on ranking the pooled data" (Quinn, 2002, p. 195).

Since there were a large number of data points, the p-values produced by the tests were extremely small. However, the actual differences in the medians were minor. This made me concerned about whether the differences were indeed significant. It is one of the classic problems with a large sample size that with a "very large sample size", some "trivial effects can produce a significant result" (Quinn, 2002, p. 51–52).

One way to compensate for such unreliability was to test the data sets again using a box-cox transformation. Eventually, the differences were more evident in that way, although the p-values remained small ($p < 0.001$). These statistical analyses and the results were also consulted and confirmed with a biostatistician through the Oslo Centre for Biostatistics and Epidemiology (OCBE) service at the Faculty of Medicine, University of Oslo.[6]

Even though the statistical results had to be interpreted carefully, the data

[6]https://www.med.uio.no/imb/english/research/centres/ocbe/

Number of samples (n)	Entropy value	Entropy max value
360	5.266	5.886

Table 4.1: Entropy test for randomness of the numeric sequences used for VP condition.

comparison suggested some differences between the conditions. However, the validity of the statistical significance remains uncertain. At this point, it was essential to be reminded about basic cell biology and the experiment design. For example, minor changes in the data could indicate larger differences in cellular responses. Moreover, the time frame I allowed for the experiment was relatively short, and data collecting time was only at one fixed arbitrary point after the stimulation. Therefore, the duration of the stimulation and data collection time could have been altered and explored further.

The impact of the stimuli and the structural alterations in cells (F-actin filaments) that seemed to be only minor provided a good statistical challenge. For these reasons, the statistical outcome had to be taken with a grain of salt. This is elaborated more fully in Paper IV.

4.4.5.2 Statistics on pseudorandom number sequences

Once the random number sequences were generated for the VP condition, I tested how much the sequence varied over time. This is partly reported in Paper IV. By performing autocorrelation tests on the sequences, I could confirm that the correlation of successive numbers fell within the 5% confidence interval and was not highly correlated. Thus, the chance of the sequence being random was statistically significant. Additionally, entropy was calculated to test the randomness of the sequence. The maximum entropy value was calculated by computing the maximum probability of the sequence. The closer the entropy value is to the maximum value, the higher the randomness of the sequence.

4.5 Phase 4: Optimisation of the stimulus-response for targeted cell culture through iterative process

The development of the experimental stimuli through various characterisations of the sound generation system, acoustically controlled environment, biological assays, and data collection methods helped to run a few preliminary experiments. Eventually, the experimental setup and protocol were developed and characterised for cell cultures. The direct research outcome of this multi-phase approach is presented in Paper IV.

For the preliminary experiments, the cell cultures were taken out of the cell incubator (typically at 37°C and 5% CO_2 level) for 15 minutes during the experiments. This was still acceptable for HeLa cell cultures but, eventually, not an ideal protocol for primary cell lines. It would be more optimal for biologists

to have the cells in the cell incubator as much as possible. For this reason, the mini-acoustic chamber was not included in the experiments for now, as reported in Paper IV. The material used was not ideal to be placed in the cell incubator (warm, high-humidity, and sterile environment) in the long run. In spite of that, the design, construction, and characterisation of the mini-acoustic chamber are still useful for other small-scale sound or radio antenna equipment measurements and tests. In addition, the experience gave me more insight into acoustics, which will help optimise the stimulus-response system for the cells. It could be modified or simplified to be used in more biologically optimised and sterile conditions, such as inside the cell incubators.

4.6 General summary and reflection

In this chapter, the multi-phase experimental methodology employed in this dissertation was presented. Generally speaking, the main factor that nourished the evolving methodology for this dissertation was the interdisciplinary dialogues, as elaborated in Chapter 3. The dialogues included not only the formal and dialectical discussions taken place in formal project meetings with the ABINO team but also through general meetings and many "corridor" conversations with the biologists and physicists at the lab facilities. Through such connections, I could vision clearer directions. Being at the labs and talking with my biology, physics, and biostatistician colleagues about research problems and solutions was an important part of the methodology in that sense.

Moreover, working hands-on in the laboratories was also a form of dialogue that helped me gain insight into the different perspectives of my ABINO colleagues. As a result, the methodology was largely inspired by the understanding of differences in research methods between the three disciplines (biology, music technology, and physics) combined in the ABINO project:

- Music technology: Generating sound stimuli through digital signal processing using music technological concepts and tools
- Music technology & physics: Developing and characterising an experimental setup
- Biology: Maintaining cell cultures in the laboratory
- Biology: Collecting meaningful data from the cells (various biological assay protocols and microscopic imaging techniques)
- Biology & biostatistics: Analysing and quantifying the biological data
- Biology & physics: Interpreting and further analysing the biological outcome

I could have stayed in the research area where I would feel more comfortable with regard to methodology. However, as a reflection, diving into other fields not only helped me to understand other fields better but also to see my own from a different angle in terms of its extendability and relevance other than musical use.

Chapter 5

Research summary

5.1 Introduction

In this chapter, the four papers included in this thesis will be presented and summarised. They are presented chronologically following the multi-phase approach discussed in the previous chapter. Although the development and investigation for each paper often occurred concomitantly, the content of each preceding paper informed the following ones. For example, the systematic literature review in Paper I provided the foundational framework that set a clearer direction for the rest of the papers in terms of filling gaps in the literature, what the cells can be stimulated with, and how the experimental apparatus should be set up. The theoretical and practical works presented in Paper II and Paper III provided more insight into the stimuli and experimental setup and helped review the design of the experiments employed in Paper IV.

5.2 Papers

5.2.1 Paper I

Reference: Kwak D., Combriat T., Wang C., Scholz H., Danielsen A., & Jensenius A. R. (2022). Music for cells? A systematic review of studies investigating the effects of audible sound played through speaker-based systems on cell cultures. *Music & Science*, 5.

Abstract There have been several studies investigating whether musical sound can be used as cell stimuli in recent years. We systematically searched publications to get an overview of studies that have used audible sound played through speaker-based systems to induce mechanical perturbation in cell cultures. A total of 12 studies were identified. We focused on the experimental setups, the sounds that were used as stimuli, and relevant biological outcomes. The studies are categorized into simple and complex sounds depending on the type of sound employed. Some of the promising effects reported were enhanced cell migration, proliferation, colony formation, and differentiation ability. However, there are significant differences in methodologies and cell type-specific outcomes, which made it difficult to find a systematic pattern in the results. We suggest that future experiments should consider using: (1) a more controlled acoustic environment, (2) standardized sound and noise measurement methods, and (3) a more comprehensive range of controlled sound parameters as cellular stimuli.

Discussion In this article, the literature on mechano-acoustic stimulations of cell cultures was systematically reviewed following the PRISMA[1] guideline. This review helped discern differences in the theoretical foundations, methodologies, and experimental findings in the available literature through the eyes of different disciplines, including biology, music technology, and physics.

The focus of the paper was on experimental studies where audible sounds or music were used as mechano-acoustic stimuli for different types of cell cultures, including mammal and bacteria cells. The study was unique because many reviews were available on plant cell cultures but not on other cell types in the specific experimental setting (i.e., stimulating cell cultures with audible or musical sounds through a speaker-based system). In addition, the literature was studied with cross-disciplinary expertise. From a physics perspective, expertise in acoustics helped to understand the physical mechanisms of the stimulations on the cell cultures. From a biology standpoint, the importance of clinical application and cell biology research was pointed out. Lastly, through a music technological viewpoint, the issues with correlating cellular response to music and possible problems with the experimental setup and environment using a speaker-based system were raised.

The two primary findings of the study were 1) the lack of acoustic control of the experimental setup and 2) the need for the development of synthesis models to systematically study different types of sonic qualities. These findings, together with other subsidiary findings, helped to develop the idea for further studies that had an influence on the subsequent papers (Paper II, Paper III, and Paper IV).

5.2.2 Paper II

Reference: Kwak D., Olsen, P. A., Danielsen, A., & Jensenius, A. R. (2022). A trio of biological rhythms and their relevance in rhythmic mechanical stimulation of cell cultures. *Frontiers in Psychology*, 13.

Abstract The primary aim of this article is to provide a biological rhythm model based on previous theoretical and experimental findings to promote more comprehensive studies of rhythmic mechanical stimulation of cell cultures, which relates to tissue engineering and regenerative medicine fields. Through an interdisciplinary approach where different standpoints from biology and musicology are combined, we explore some of the core rhythmic features of biological and cellular rhythmic processes and present them as a trio model that aims to afford a basic but fundamental understanding of the connections between various biological rhythms. It is vital to highlight such links since rhythmic mechanical stimulation and its effect on cell cultures are vastly underexplored even though the cellular response to mechanical stimuli (mechanotransduction) has been studied widely and relevant experimental evidence suggests mechanotransduction processes are rhythmic.

[1] https://prisma-statement.org/

Discussion This is a perspective article that formed an important part of my theoretical framework for this dissertation. This paper is an amalgam of perspectives from biology and musicology.

The paper provides an overview of how rhythmic biological processes span over a wide range of temporal scales, which can be from microseconds to several hours and days. With a short introduction to biological rhythms and different levels of rhythms—ultradian, circadian, and infradian—the paper lays out why it may be crucial to think of cellular processes as rhythmic phenomena. For example, the paper proposes a rhythm model consisting of central, external, and reflexive or consequential rhythms. The discussions around the model include how these three components are interconnected, hence the name "trio". The discussion also points out how the connection is critical for biological systems to maintain their homeostasis.

The paper accentuates the need to explore rhythmic stimulation more in *in vitro* cell models where mechanical probes are used. Moreover, this paper encourages researchers to consider effects of the rhythmic stimulation of biological systems in the context of experimental mechanobiology research.

5.2.3 Paper III

Reference: Kwak D., Krzyzaniak M. J., Danielsen A., & Jensenius, A. R. (2022). A mini acoustic chamber for small-scale sound experiments. *AM '22: Proceedings of the 17th International Audio Mostly Conference.*

Abstract This paper describes the design and construction of a mini acoustic chamber using low-cost materials. The primary purpose is to provide an acoustically treated environment for small-scale sound measurements and experiments using $\leq$ 10-inch speakers. Testing with different types of speakers showed frequency responses of $<$ 10 dB peak-to-peak (except the "boxiness" range below 900 Hz), and the acoustic insulation (soundproofing) of the chamber is highly efficient (approximately 20 dB SPL in reduction). Therefore, it provides a significant advantage in conducting experiments requiring a small room with consistent frequency response and preventing unwanted noise and hearing damage. Additionally, using a cost-effective and compact acoustic chamber gives flexibility when characterizing a small-scale setup and sound stimuli used in experiments.

Discussion In this paper, I aimed to tackle the problem of inadequate control of the sonic environment of cell cultures. I built an acoustically treated chamber to contain cells and speakers to stimulate cell cultures with sound in an acoustically controlled environment. The chamber can also function as a practical and portable measurement and testing environment. For that purpose, the mini chamber, as opposed to a full-size anechoic chamber, which can be costly, was essentially a small rectangular box treated with in-expensive acoustic materials accessible at local stores.

5.2.4 Paper IV

Reference: Kwak D., Combriat T., Krauss, S., Jensenius, A. R., & Olsen, P. A. (2022). The effect of rhythmic vertical vibration of cell culture on the F-actin filament structure. (Will be submitted soon.)

Abstract This paper aims to provide an experimental protocol involving a rhythmic vertical vibration setup controlled by music technological concepts and tools. We characterize the experimental setup using 3-axis acceleration measurement and Particle Tracking Velocimetry (PTV) method. We report this in terms of RMS acceleration, fluid flow patterns, and estimated shear forces generated in the cell plate wells, which have not been investigated before in similar vibration studies. The rhythmic experimental conditions included No Vibration (NV), Continuous Vibration (CV), Regular Pulse (RP), and Variable Pulse (VP). As a functional readout, the F-actin filament structures were analyzed through fluorescence microscopy and a feature extraction algorithm we developed. Our data show a general tendency of size (length and thickness) reduction and altered angle (2D orientation) of the filaments in all vibrated cell cultures. We also found accumulations of cells in the G1-phase in the vibrated cell cultures through an EdU proliferation assay. The effects were more apparent under CV than RP and VP. Consequently, compared to the magnitude of the mechanical parameters, we report a negative correlation with F-actin filament structural properties and a positive correlation with the accumulations of cells in the G1-phase of the cell cycle. These analyses inspire further investigation of the possible effect(s) of rhythmic mechanical cues on cell cultures.

Discussion The outcome of the literature review, and the theoretical, practical, and experimental considerations in the previous papers included in this dissertation, culminated in this experimental study. Accordingly, this paper can be considered a product of the multi-phase approach presented in Chapter 4.

In this paper, HeLa cell cultures were mechanically stimulated with a vertical vibration generator that was controlled by audio signals designed and generated in Max/MSP. Apart from the temporal regularity of the four experimental conditions (NV, CV, RP, and VP), the frequency, amplitude, and waveform of the signal were kept the same. The rhythmic conditions (different pulse durations and intervals) were created by interrupting the digital signal flow (gate) at varying time intervals.

The experimental setup was characterised thoroughly by measuring the acceleration (g) and estimating the shear stress (N/m^2) through the particle tracking velocimetry method. Although the acceleration and shear stress in experimental models has been previously investigated many times in different context, there has not been many studies that investigated the possible effect of shear stress in a vibration system coupled with cell cultures.

The data produced from the experiments reported in this paper suggest that the structural properties (length, thickness, and angle) of the F-actin filaments in vibrated HeLa cell cultures altered compared to the non-vibrated cell

cultures. The differences observed in between different experimental conditions suggest that the structural differences were more prominent under CV than RP and VP. The data also suggest that the cells responded differently between RP and VP. Therefore, the empirical outcome of the paper suggests that mechanical stimulation with a short-term temporal variation can affect the dynamic structuring of the F-actin filaments of HeLa cells differently.

As the paper's main purpose was to report the establishment of an experimental protocol that is well-characterised for reproducibility and reliability, there was a limited time to study the biological outcome more in-depth. This paper calls for a further investigation to test whether the differences observed are indeed reproducible in HeLa cells (and possibly other cell lines) and investigate the mechanisms behind the differences observed between CV-RP, CV-VP, and RP-VP.

Chapter 6

Discussion

*"If you take a rock and toss it into the air, it traces out a nice parabola.
If you take a bird and throw it into the air, it flies off into the trees somewhere.
There's an awful lot of internal information processing going on, [...]
that's true even if you go down to simple cells.
They aren't just responding to simple forces."*
– Waldrop, 1992, p. 232

6.1 Summary: Addressing the research question

In this chapter, I would like to begin with a discussion of the results and insights gained from the papers to address the research question laid out in Chapter 1. A general discussion, suggestions for future work, and reflection on interdisciplinarity will be presented afterwards.

In order to address the main research question, a multi-phase approach was taken. This approach provided space and time to:

- investigate the literature and develop a theoretical framework and perspective presented in Chapter 3, Paper I, and Paper II
- design experimental procedures, perform experiments, refine data collecting methods, and analyse and interpret the collected data presented in Chapter 4, Paper III, and Paper IV

In the following sections, the sub-questions are discussed to answer the main research question.

6.1.1 RQ1: Can the cells sense audible sounds?

The theoretical considerations in Chapter 3 and Paper I illuminated that cell cultures (i.e., *in vitro* cell models) most likely do not sense audible sounds directly. However, cell cultures could sense other mechanical stress induced by audible sounds. This mechanism may be feasible through the induced fluid flow within the wells of the cell culture plate by the vibrations generated through audible

sounds. This supposition is not necessarily encouraging or discouraging. It instead broadens the perspective.

Generally, life sciences research evolves around mimicry of the natural *in vivo* microenvironment. When it comes to sound sensing mechanisms found in nature, I found the human auditory system to be the prime biological model. This may sound too obvious, but it has not been the centre of attention in the field of mechano-acoustic stimulation of cell cultures. The mechanism involves the conversion of the sound pressure waves into the movement of the liquid in the cochlea, where hair cells can be found.

Similarly, the vibration system controlled by audio signals used in this dissertation induced a liquid flow inside the cell culture plate. The cells were attached to the bottom surface of the cell plate. As a result, the fluid flow could have generated shear stress on the cells. The effect of shear stress on cell cultures has been shown through different experimental apparatus, such as microfluidic systems, previously (De Belly et al., 2022). Therefore, it is not far-fetched to hypothesise that the cell cultures could indirectly sense or be affected by the audible sound vibrations through induced shear stress. Although the cell models employed in this dissertation are non-auditory cells, this conversion of sound vibration to fluid flow can be an imitation of the natural biological system.

It is also possible that the cell culture plate resonates when sound is played directly to it. The resonance in the cell plate surface is a factor that contributes to inducing fluid flow. However, the surface waves induced through audible sounds can be complex, affect the cell culture plate globally, and are not as controlled and narrowly focused as when ultrasound is used. Further investigation is needed to confirm how large and complex surface vibration waves can impact cell cultures.

With this in mind, in this dissertation, the alterations in the F-actin filaments of vibrated cell cultures were used as a functional readout to discern whether the cells were sensing and responding to mechanical stresses. This was because the F-actin filaments are part of the cellular components responsible for the mechanotransduction process, and their responding time can be rapid (Ingber et al., 2014; Priya and Yap, 2015). The fluorescent staining and imaging under a high-resolution fluorescence microscope were performed to visualise the F-actin filaments in cells. The feature extraction from the collected image data suggest alterations in the length, thickness, and angle of the F-actin filaments after the cell cultures were vibrated.

Collectively, within the experiment designed and performed in this dissertation, the data suggest that the organisation of the F-actin filaments of the cells altered after being exposed to vertical vibrations of the speaker-based vibration system driven by the audible sounds. In this way, the cell cultures may have sensed audible sound vibrations indirectly, possibly through shear stress generated by the induced fluid flow.

6.1.2 RQ2: What parameters of musical sounds are controllable and relevant to cell cultures?

The systematic literature review in Paper I pointed out that previous studies primarily focused on using and altering sound amplitude, frequency, waveform, and duration of the stimulation to generate mechano-acoustic stimuli. In this dissertation, along with these essential sound parameters, the main focus of the study has been rhythm, defined as "a series of events that regularly varies and repeats, generating patterns in time".

From a music theory perspective, there are many other features to consider: harmony, melody, timbre/texture, spatiality, form, etc. However, the insights gained from the first two papers (Paper I and Paper II) point to rhythm as a highly relevant element in both biology and music technology. The key point derived from the two papers is that rhythm in biological and musical systems is an observable and measurable phenomenon, and it is an important element as an indication of a functioning dynamic system. Thus, the outcome of the two papers had a major impact on the decision to explore rhythm as an experimental variable.

In the experimental method developed in Paper IV, rhythm as an experimental parameter was employed as Regular and Variable Pulse conditions. The temporal aspects of these two mechano-acoustic conditions were controlled using numerically strict or varied pulse durations and intervals, while amplitude, frequency, waveform, and duration of the stimulation time were kept the same. Such temporal variations that could mimic the dynamic nature of biological systems can be highly relevant in life sciences research, where imitation of the natural environment is a central question.

Taken together, within the framework of this dissertation rhythm was considered the most controllable and relevant parameter.

6.1.3 RQ3: What is the effect(s) of (musical) sounds on cell cultures?

This question was investigated by studying the alterations in the F-actin filaments of vibrated HeLa cell cultures under four different experimental conditions: No Vibration (NV), Continuous Vibration (CV), Regular Pulse (RP), and Variable Pulse (VP). To answer this sub-question, I find the following three categories of the experimental conditions based on the data from Paper IV important to consider:

Vibrated vs non-vibrated: The length and thickness of the F-actin filaments of cells are generally reduced in vibrated conditions compared to the non-vibrated condition. The angles (2D orientation) of the F-actin filaments of the vibrated cells were also altered compared to the non-vibrated cells.

Rhythmic vs non-rhythmic: Cells responded differently to rhythmic (RP and VP) and non-rhythmic (CV) vibration conditions. The size of the F-actin filaments of the cells treated with the rhythmic conditions did not shrink

as much as the ones treated with the non-rhythmic condition. The angles of the F-actin filaments under the rhythmic conditions also did not alter as much as the ones under the non-rhythmic condition.

RP vs VP: Differences in cellular responses are also noticed between the two rhythmic conditions. The cells treated with RP had similar filament lengths but smaller filament thicknesses than those treated with VP. RP and VP conditions also produced differences in the angles of the filaments. The angles of the F-actin filaments under RP condition were more similar to the ones under non-vibrated than that of VP.

Taken together, the F-actin filaments of the vibrated cells generally reduced in size, and the data suggest that the "reducing effect" was more apparent under CV than RP and VP.

Furthermore, the data suggest that the cells under CV and RP higher number of cells compared to the control (NV) in the G1-phase of the cell cycle distribution, which could mean the cells are going under apoptotic pathway or DNA repairing mechanisms (Murad et al., 2016). However, the data collected from the proliferation experiment (Incucyte analysis: a real-time live cell imaging) suggest that all the conditions reached the full confluence without any significant alterations in the expected growth rate of the cell culture. This is an indication that the cells are not going under the apoptotic pathway (cell death), but whether the cells were going under DNA repairing mechanisms needs to be investigated further.

Through characterisation of the experimental setup, it was shown in Paper IV that the rhythmic conditions had a shorter total amount of vibration time, lower RMS acceleration, and lower shear stress than non-rhythmic conditions. This could be simply interpreted as the longer the stimulation, the more severe the effects (reduced F-actin filament size and increased number of cells in the G1-phase). This is also reported in Paper IV, where the analysis suggests a strong negative correlation between the mechanical parameter magnitude and the effects observed in the cells. Through the statistical analysis of the data, we also found that the longest and thickest F-actin filaments (a few extreme outliers) were all found in rhythmic conditions. Especially, the VP condition produced the longest and thickest F-actin filaments.

Although the reliability of the image processing and feature extraction has been validated through various tests (Chapter 4), the extreme outliers can be random incidences found in the data and can complicate the findings. Nonetheless, cells have complex processes, and the outliers (longest and thickest filaments) can indicate that rhythmic conditions affected the cells to produce more stress fibers, which are highly organised bundles of actin filaments, and their ends can be linked at the focal adhesion points responsible for sensing extracellular mechanical cues (Katoh et al., 2001). Stress fibers form in response to the applied mechanical strain, and the destabilisation of stress fibers can inhibit the mechanoadaptation of the cells (Roshanzadeh et al., 2020). Therefore, the differences observed should be cross-checked through other biochemical assays (e.g., through gene or protein

expression levels related to the F-actin filament polymerisation processes). Also, further investigation is needed to verify the mechanisms behind the outcomes of our experiments and the ways in which the rhythmicity (i.e., regularity of the pulse durations and intervals) of the mechanical stimulation of the cell culture might have affected the cellular response.

In sum, the data show discernible effects of audible sounds on the cell cultures in terms of the F-actin filament structures (reduced filament length and thickness and altered angle) and cell cycle distribution (increased number of cells in G1-phase). However, the underlying biological mechanism remains elusive.

6.2 General discussion and conclusion

I aimed to investigate how sound parameters and musical elements can be used and manipulated to produce beneficial alterations in cell cultures. This investigation was part of the ABINO project's goal to find out whether playing (musical) sounds can have any effect on cell cultures in a controlled laboratory environment through the interdisciplinary endeavour.

Accordingly, the theoretical discussions in this dissertation addressed some of the fundamental questions arising from different disciplines combined in the project. Especially, I tried to cast some light on how the cells can sense and respond to mechanical alterations in their microenvironment and how that could help design the experiment studying the cellular responses to sound stimuli. After learning about some of the important cell sensing mechanisms, one of the central parts of the study has been investigating what sounds to play to the cells.

On that note, I found a fascinating connection between cellular and musical systems, which is rhythm. This is not to claim that cellular or biological systems are musical or musical systems are based on biology: though it may be the case. At least, it has been made more apparent that many of the measurable biological signals (e.g., from the cells) can be rhythmic (Paper II). As indicated in Paper II, there is evidence of the effect of rhythmic mechanical stimulation on rhythmic cellular responses and regulations of specific genes (e.g., CLOCK) that are crucial in keeping the internal cellular rhythm (Rogers et al., 2017; Vágó et al., 2022). It appears that rhythm may be an essential element that can be observed in the organisation of the biological activities in cells, and it is interestingly an essential element in organising sounds in music too.

In addition, this dissertation described how a life sciences research project involving cell biology, music technology, and physics can be combined to produce an experimental setup and protocol to study the mechanical properties and responses of cell cultures. The methodological approach, which was a fusion of perspectives from multiple disciplines, was largely inspired by my interdisciplinary group of colleagues from at various departments and research centres at the University of Oslo. The in-depth considerations of interdisciplinary issues and questions have helped this dissertation tremendously in many different ways. In line with that, the dissertation included a theoretical framework for the concept of

radical interdisciplinarity and practical considerations regarding interdisciplinary research, which is reflected upon in the later sections of this chapter.

Finally, some of the general conclusions of this dissertation include the following:

Cellular "perception" of audible sounds, including music: Cells have sensing mechanisms to gather external information from their immediate microenvironment. This is crucial for their optimised function and survival in their environment. Cells can theoretically sense sound vibrations, but whether audible sounds can have a direct impact on the cell cultures are unclear due to the physical limitations. However, audible sounds can induce fluid flow to generate shear stress on cell cultures, which could be comparable to the sound perception mechanism employed in the human auditory system. This mechanism could explain why musical sounds may indirectly affect cell cultures and connect the research problem to fluid dynamics and/or microfluidics since it has been widely known that cells can sense and respond to shear stress.

Cellular response to audible sound vibrations: The experimental data produced by this dissertation show reduced F-actin filaments size and the tendency of higher number of cells in the G1-phase cell cycle in vibrated cell cultures. The effect was more evident under the non-rhythmic condition than rhythmic conditions. The results also show that F-actin filament structural properties are negatively correlated ($r < -.9$), and the number of cells in the G1-phase cycle is positively correlated ($r > .9$) in relation to the magnitudes of mechanical parameters (RMS acceleration and shear stress). A simple explanation could be that the total amount of time that rhythmic conditions vibrated the cell culture was shorter in the rhythmic than the non-rhythmic condition, affecting the RMS acceleration and magnitude of the estimated shear stress the cells may have experienced.

However, there are some confusing statistical results in the data that requires further investigation. For example, the data indicates that while the cells under the rhythmic conditions produced shorter median length and thickness, they also produced several of the longest and thickest F-actin filaments than the control. This can be data points that should be filtered out (extreme outliers), but it may also contain biological implications that can be relevant, such as stress fibers. Therefore, a more in-depth functional analysis of the vibrated cell cultures is necessary.

The accumulation of cells in the G-1 phase could indicate that the cells were going under apoptotic pathways or DNA repair mechanisms. However, since all the vibrated cell cultures reached the full confluence and showed the expected proliferation rate, the data do not suggest that the cells were going through apoptosis. Whether the accumulation is due to the DNA repair mechanisms is unclear, and this also needs to be investigated further.

Radical interdisciplinarity: A combination of disparate and incommensurable disciplines in a problem-oriented research project was successfully carried

out to produce a synergistic—“an effect that is greater than additive”[1]—outcome that includes an interdisciplinary theoretical framework and experimental design. This was possible through many forms of dialogues (e.g., agreeable and disagreeable, constructive and critical, formal and informal, and theoretical and practical) that helped to internalise the fundamental questions and ways of thinking between the disciplines.

Insights from this dissertation call for more systematic investigations of cellular responses to rhythmic stimuli. This could be more directly related to chronobiology research. However, the call is for more specific areas of life sciences research, for example, rhythmic mechanical manipulation of cellular processes concerning more primary cell lines, such as hiPSCs.

6.3 Future Research

Musical sounds: A systematic investigation of more sophisticated musical signals is still needed. Originally, my plan was to work with various levels of sonic complexity in the stimuli. That was not possible due to time limitations. Such studies can involve, for example, more than one frequency, different sonic texture qualities, and a fully composed piece that has more meaningful musical content.

Rhythmic stimuli: The design of the rhythmic signal could be explored more, as there are many possibilities regarding how time can be organised through digital signal processing techniques (Wakefield and Taylor, 2022). Rhythm has gained popularity as a research topic in the mechanobiology and tissue engineering paradigm only in recent years. Thus, only a few investigations have been addressing the topic. Rhythmic mechanical stimuli could create a microenvironment closer to the condition mimicking the biologically relevant and naturally dynamic environment. Rhythm is a critical gap that needs to be filled in life sciences research. Therefore, investigating and exploring rhythmicity in the *in vitro* cells would be a valuable contribution to the field.

Variable Pulse (VP) condition: This condition could be developed further and designed to mimic more dynamic or natural temporal fluctuations (the current VP condition is based only on a pseudorandom number sequence), for example, temporal cues that obeyed long-range correlation (Torre et al., 2013). This is contrasted to Continuous Vibration (uninterrupted mechanical stress) and Regular Pulse (mechanical stress at rigidly the same rate) conditions investigated in this dissertation.

The long-range correlation condition shows a slow decay in the correlation between succeeding points over time, as opposed to the short-range correlation condition that shows a rapid decay in comparison or white

[1] https://www.oed.com/view/Entry/196480

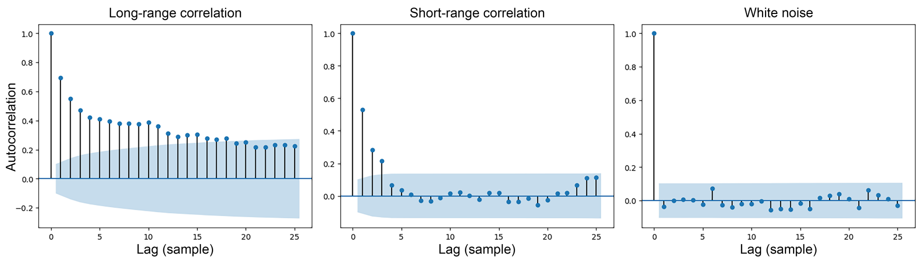

Figure 6.1: A comparison illustrating the differences between the autocorrelation of long-range correlation, short-range correlation, and white noise.

noise where there is no correlation, as illustrated in Figure 6.1. Long-range correlation is known to be found in numerous complex biological systems, such as in DNA sequences (Peng et al., 1992), natural language (Tanaka-Ishii, 2018), and bird songs (Markowitz et al., 2013). Torre et al. (2013, p. 342) stated that "rhythms generated by natural bio-behavioral systems exhibit a characteristic structure of fluctuations over time, namely long-range correlation, or fractal fluctuations".

This temporal model has already been developed and tested in a different project at RITMO where the temporal stimuli (numeric sequences) were generated in the R statistical programming language and imported in the Max/MSP environment, as shown in Figure 6.2. The time sequence was generated by using the *fracdiff* package in the R (https://github.com/mmaechler/fracdiff). The model could be easily modified to be used in life sciences research.

Cellular responses to rhythmic stimuli: The development of experimental protocols to analyse cellular rhythms, such as regulation of CLOCK, PER, CRY genes (Vágó et al., 2022), would greatly help to understand the different cellular responses to non-rhythmic and rhythmic mechanical stimuli.

Live imaging: There are available methods to image the GFP-labelled cells live under a fluorescence microscope while they are perturbed mechanically. For example, live imaging while directly manipulating the cytoskeleton mechanically using the atomic force microscopy technique has been previously demonstrated (Li et al., 2020). As far as I understand, live imaging is not the most straightforward setup to work with, but observing the cell structures during the stimulation could produce data that can explain clearly how the cells sense and respond to the stimulus and whether or not it can be "beneficial" for the cells.

Primary cell line: The effect of rhythmic mechanical stimulation on more primary cell lines, such as hiPSCs, is still needed. The current study used only HeLa cells, which are easier to work with than other cell types.

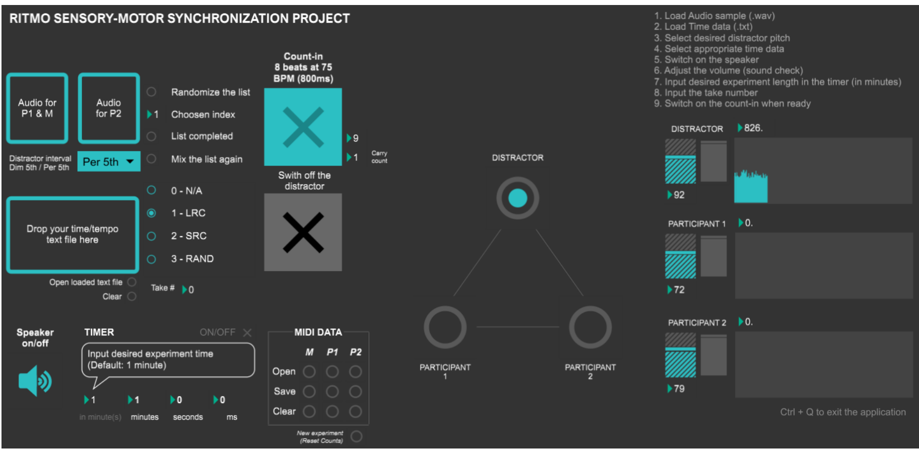

Figure 6.2: The Max/MSP user interface to test temporal stimuli following long-range and short-range correlation models, developed and designed in the R and the Max/MSP environment.

However, the model has flaws since it is derived from cancerous cells and can show high chromosomal instability (Landry et al., 2013). The investigation into primary cell lines would produce higher-quality data and positively impact the field.

Embedded system: The development of an embedded sound vibration system with an interface to control signals could enhance the practicality of the experimental setup. The whole experimental system could be made portable and placed into an incubator safely with a monitoring system that can disregard the need to connect to a function generator or laptop.

Machine learning: Implementing a machine learning algorithm could benefit the experimental protocol. Although the data acquisition can be expensive and not abundant in numbers, an algorithm could be used to optimise the stimuli (e.g., manipulation of the digital audio signals) to enhance the targeted biological responses from the targeted cell cultures.

6.4 Interdisciplinary reflection

This dissertation began with a discussion on radical interdisciplinarity (Chapter 2), and here at the end, I would like to circle back and reflect on the concept that has been employed throughout this dissertation.

6.4.1 Different levels of disciplinarity

In Chapter 2, I discussed Holbrook's interdisciplinary communication model (Holbrook, 2013) and questioned how far one can push to truly internalise

(make it one's own) concepts and methods of disciplines other than one's "home" discipline.

In my PhD project, I found myself somewhere in between interdisciplinary and transdisciplinary, as illustrated in Figure 2.1. I got my hands "dirty" in other disciplines, so to speak. This is because the ABINO project provided an opportunity for senior researchers and PhD students from various fields to sit around a table and bring each field's perspectives and approaches. As far as I am concerned, the project has been successful and also paved the way for other novel research possibilities and developments.

Having considered disciplinarity at different levels, to have a rigid dichotomy about whether one is an interdisciplinary researcher is tricky, and many researchers will find themselves anywhere between cross- and transdisciplinary in Figure 2.2. As I exit out of the project, I had to ask what disciplinary/ies identity/ies I hold. In the following section, I will discuss this point further.

6.4.2 Disciplinary identity: Individual researchers and disciplines

During my studies, I asked this question often: at the individual level, should one strive for interdisciplinarity or even transdisciplinarity? In my opinion, this is a decision that should be left up to the individual researcher. It also depends on the project one is working within and the other researchers involved. I do not think it is either good or bad to be at a certain level of disciplinarity. In many cases, there may even be combinations within one research group: some may be working more intradisicplinary while others strive for transdisciplinarity.

One important consideration is whether one identifies as a researcher participating in an interdisciplinary project or as an interdisciplinary researcher. In the first case, although the person participates in an interdisciplinary project and contributes by offering skills and expertise, the core identity could stay the same. Hence, there is no real reception of other disciplines, and the researcher's disciplinarity does not change. This can be voluntary. For example, the person can choose not to invest time in adopting or inventing a whole new system of thoughts and methods.

The level of disciplinarity can also be somewhat "involuntary". For example, it may be difficult to find a journal that accepts an interdisciplinary manuscript. Many people have also argued that a discipline's publication principles can affect one's academic career possibilities (Domino et al., 2007; Lariviere et al., 2006). Such an "interdisciplinary dilemma" (Domino et al., 2007) may inhibit the individual researcher from exploring interdisciplinary perspectives.

My starting point was that of music technology, which on its own is an ill-defined field comprising researchers from the arts and humanities, design, computer science, and engineering. Through my PhD work, I have combined this with rhythm studies, biology, and physics. This PhD is submitted to the Faculty of Humanities in music technology, but I think it is inspiring to be a transdisciplinary piece of work. Throughout my research, I have moved from category to category in Jensenius' illustration (Figure 2.2). Time will tell whether this mixture can loop back to intradisciplinarity one day.

6.4.3 An interdisciplinary vision

Can a research group, department, or faculty do interdisciplinary or transdisciplinary research? Can a discipline leave its territory and be flexible enough to adopt a whole new concept, theory, and method and transform itself into a new hybrid discipline? Should individual disciplines settle for "side projects" where other disciplines can join and leave afterwards (e.g., borrowing concepts, theories, and methods from other disciplines)?

To provide a perspective to that question, interdisciplinary research centres, such as RITMO and HTH at the University of Oslo, play an essential role in creating suitable environments. In my experience, these centres allow for wider disciplinary boundaries to facilitate cross-fertilisation between different disciplines and breed more "free-range" hybrid disciplinary researchers and projects. Indeed, this work would not have been possible without such centres, and I strongly feel that interdisciplinary visions can be put into practice more "safely" and effectively in these environments.

Despite the tendency of this dissertation towards transdisciplinarity, I still believe the work contributes to my "home" field, namely music technology, and belongs to the Faculty of Humanities, where I have followed doctoral training. As Wakefield and Taylor (2022, p. 1) writes, this work demonstrates that a few simple musical signal processing techniques can be used to explore "the immense possibilities of digital signal processing" and its implementation. I hope that the efforts made to develop the experimental protocol for studying cellular responses from the perspective of music technology can inspire others in the same field.

Last but not least, whether interdisciplinary projects can produce real innovation and useful outcomes is an important question. Within the framework of this dissertation and also the ABINO project, a combination of perspectives and methods culminated in an experimental design that provided a new angle or approach to study cellular response: rhythmic mechanical stimulation of cell cultures, which is underinvestigated, using music technological concepts and tools.

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Papers

I

Paper I

Music for cells? A systematic review of studies investigating the effects of audible sound played through speaker-based systems on cell cultures

Dongho Kwak, Thomas Combriat, Chencheng Wang, Hanne Scholz, Anne Danielsen, Alexander Refsum Jensenius

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Music for Cells? A Systematic Review of Studies Investigating the Effects of Audible Sound Played Through Speaker-Based Systems on Cell Cultures

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Abstract

There have been several studies investigating whether musical sound can be used as cell stimuli in recent years. We systematically searched publications to get an overview of studies that have used audible sound played through speaker-based systems to induce mechanical perturbation in cell cultures. A total of 12 studies were identified. We focused on the experimental setups, the sounds that were used as stimuli, and relevant biological outcomes. The studies are categorized into simple and complex sounds depending on the type of sound employed. Some of the promising effects reported were enhanced cell migration, proliferation, colony formation, and differentiation ability. However, there are significant differences in methodologies and cell type-specific outcomes, which made it difficult to find a systematic pattern in the results. We suggest that future experiments should consider using: (1) a more controlled acoustic environment, (2) standardized sound and noise measurement methods, and (3) a more comprehensive range of controlled sound parameters as cellular stimuli.

Keywords

Mechano-acoustic stimuli, sound stimuli, music stimuli, cell cultures, mechanobiology

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Introduction

Is there such a thing as *music for cells*? The use of musical sound to induce a beneficial physiological and psychological effect on biological beings is not a novel idea (Babikian et al., 2013). In light of this, the efforts to attain a deeper understanding of the relationship between sound and the human body have become more specific, now at a minuscule level in the field of mechanobiology in the last few decades. Mechanobiology has developed substantially with more than 33,000 publications up until 2016 (Sokabe, 2016; Wall et al., 2017). More recently, there has been an indication of escalating interests and efforts to understand mechanotransduction through mechanical cues induced by acoustical perturbation on cellular responses (Exbrayat & Brun, 2019). Various cell types have been investigated including: plant cells (Lim & Bae, 2014), micro-organisms (Algieri et al., 2018), auditory cells (Exbrayat & Brun, 2019), and other mammal cells *in vitro* (Kumeta et al., 2018) or *in vivo* (Wei et al., 2016). The effects of sound on plant cells have been reported and reviewed numerous times previously (Hamant &

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Haswell, 2017; Hendrawan et al., 2020; Jung et al., 2018; Mishra et al., 2016). Hence, the aim of this review is to get an overview of studies that have used audible sound played through speaker-based systems to induce mechanical perturbation in bacteria, yeast, and mammalian cell cultures. We start with a brief background to mechanobiology and the basic physical properties of sound. Subsequently, the selected studies are discussed concerning the experimental setups, sound used (categorized into "simple" and "complex" sounds), and experimental outcomes.

Background

The Mechanobiology of Cells

Cells *in vivo* are endlessly perturbed by different types of mechanical forces such as tension, compression, and shear stress (Mishra et al., 2017), and it has been suggested that cells are sensitive to physical forces (Kung, 2005). Mechanical signals, such as adhesiveness or extracellular matrix (ECM) stiffness, are also sensed by cells (Zhang et al., 2020). Different types of mechanical cues instigate specific responses in cells where certain genes can be activated through specific signaling pathways (Fedorchak et al., 2014). For instance, YAP and TAZ of the Hippo pathway, which has been identified as conserved mechanical transducers, change their locations to the nucleus in cells if the stiffness of the surface is rigid (Argentati et al., 2019). This nucleus localization is critical for driving stem cells' fate (e.g., targeting the cell type that stem cells change to through differentiation process; Zhang et al., 2020). At the same time, its altered activity is involved in aberrant cell mechanics transduction and several diseases (Panciera et al., 2017). Cells have different means to sense the mechanical changes in their immediate surroundings, which include mechanosensitive ion channels, membrane mechanosensors, and mechanosensitive proteins inside the cells (Yang et al., 2015). Interestingly, it has been reported that cells cannot only sense mechanical stimulations but also generate mechanical signals themselves. For example, bacterial cells can generate sound waves between 8 and 43 kHz (Matsuhashi et al., 1998), and mechanical vibrations of yeast cells (Pelling, 2004) and stem cells have also been observed (Ventura et al., 2017).

Sound as a Mechanobiological Stimulus

Sound is essentially vibration. The word vibration originates from the Latin word *vibrationem,* which means shaking. When an object is disturbed by a mechanical stimulus and starts shaking and vibrating, sound energy is produced (Wood, 1946). This energy is propagated in the form of pressure waves from the sound source, usually as a longitudinal wave consisting of compressions and rarefactions of matter (Huber, 2010). Sound waves can travel through different mediums, including gas, liquid, and solids, with speeds dependent on the medium (Nandanwar et al., 2017). Typically, sound travels faster in a liquid or solid medium (e.g., water or a steel bar) than in a gas medium (Huber, 2010; Khilyuk et al., 2000).

A medium's density impacts energy transmission when sound is traveling from one medium to another (Wood, 1946). This physical property of sound is a factor that needs attention in cellular experiments, in which sound energy transmits from the air into a liquid-based solution such as cell culture media. For a progressive plane wave, the physical property of a medium can be expressed as the acoustic impedance, defined in equation 1.

$$Z = \rho c \tag{1}$$

In equation 1, ρ is the density (kg/m^3) of a medium and c is the speed of sound (m/s) in the medium. At the interface between two media of mismatched acoustic impedance, part of the incident energy will not be transmitted but reflected. The amount of acoustic energy that will undergo reflection at the interface between a medium of impedance Z_1 and a medium of impedance Z_2 can be evaluated by the acoustic energy reflection coefficient defined in equation 2.

$$R = \left(\frac{Z_2 - Z_1}{Z_2 + Z_1}\right)^2 \tag{2}$$

For example, sound pressure waves traveling from air at 20 °C ($Z_1 \approx 413$ Pa·s/m) will be reflected almost entirely ($R \approx 0.9989$) at the surface of water ($Z_2 \approx 1.5 \cdot 10^6$ Pa·s/m).

The medium's thickness also impacts the transmitted energy. If the thickness of the second medium is much smaller than the wavelength of the incident sound, for instance, the amount of transmitted energy will be minimized (Wood, 1946).

Manipulable Sound Parameters

When exploring the use of sound for the experimental set up of cells, there are several properties to consider. Some of the basic parameters of sound include its frequency content (which contributes to the perception of pitch and timbre), amplitude (which corresponds to perceptual loudness), and exposure time. Additionally, there are different types of waveforms, from the most basic (sine tones) to synthetic waveforms (sawtooth, square, triangle), and more complex and natural waveforms (noise, speech, music) (Farnell, 2010).

When it comes to basic descriptions of sounds, the sound frequency (Hz) is represented as the number of cycles or vibrations per second. It can be defined as:

$$f = \frac{1}{T} \; or \, f = \frac{c}{\lambda} \tag{3}$$

In equation 3, T is the period (s) which is the time taken for a vibrating motion to complete one cycle, λ is the wavelength (m) which is the distance between two successive matching points in any section of the sound wave cycle.

When the period and wavelength increase, the frequency will decrease in a given material, but the speed of sound will remain the same unless the temperature changes (Wood, 1975). In the following, we will discuss the review method and selected studies.

Review Method

We searched for articles using PubMed and Google Scholar. The keywords included in the search were: "sound," "music," "acoustic stimulation," and "cell culture." The combinations of words used for the search were: "sound stimulation on cell culture," "music stimulation on cell culture," and "acoustic stimulation on cell culture." Publications were collected between July 1, 2019 and June 20, 2020.

As illustrated in Figure 1, a total of 95 records were identified through database searching. Of these, seven records were removed as duplicates, 74 records were investigated but considered not relevant and hence removed. This included studies that were not related to cell studies *in vitro*, or not related to audible sound stimulation, or publications not in English. The full texts of two records were unobtainable and were not considered either. This left us with 12 studies that were included for further qualitative consideration (Table 1).

Results

When looking broadly at the selected studies, the sound used in the literature can be divided into two main sound types:

- simple sounds (e.g., sine tones): six studies
- complex sounds (e.g., music and speech): six studies

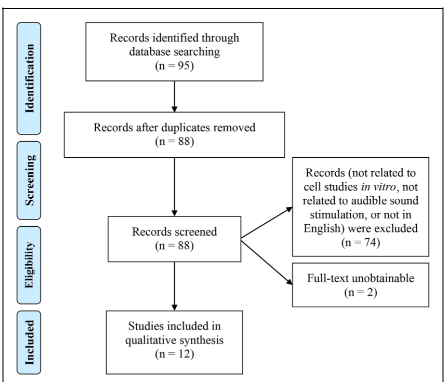

Figure 1. The PRISMA flow diagram for systematic review (search and filtering results).

We will now look more closely at each study in terms of the experimental setup, sound parameters, and biological outcomes.

Simple Sound Stimulation on Cell Cultures

The studies identified within the category simple sound stimulus all used a sine tone with a constant sound pressure level in the experimental settings. An overview of the findings can be found in Table 2.

In Shaobin et al. (2010), a speaker was placed above the petri dish inside a box-shaped temperature-controlled (37 ° C) environment. The box was acoustically treated with sound absorption around the walls. Sine tones with frequencies of 1,000, 5,000, and 10,000 Hz were played separately at 90 dB SPL for 1 h at a time and repeated six times with 3 h breaks in between. *Escherichia coli* were spread on an agar plate and were therefore not in a liquid-based media. The petri dish holder was made to rotate using a motor installed outside the chamber. It is not stated whether the control group was placed on the holder while the holder was rotating. Cell colony-forming efficiency was tested, and 1,000, 5,000, and 10,000 Hz sine tones showed upregulation of the cell colony forming by 141.6, 130, and 131.1%, respectively.

In Aggio et al. (2012), a pair of mid-high frequency speakers and a subwoofer was placed around shaker-flasks in a cell incubator. The distance between speakers and flasks was 20 cm. Sine tones of 100 and 10,000 Hz were played using computer software at 92 and 89 dBA, respectively, for 14 h to *Saccharomyces cerevisiae* yeast culture. The results showed an effect on the exponential growth rate where it was increased by 8.7% ($p<.005$) when 100 Hz sine tone was played to the cell culture and by 11.5% ($p<.005$) with 10 kHz sine tone. Subsequently, the cells exposed to sine tones showed reduction in the biomass production which decreased by 14% ($p<.05$).

In Choi et al. (2016), an 8-inch active speaker (NA-818APR, NBN) was placed underneath (10 cm away) the cell culture dish inside a cell incubator. A 1000 Hz sine tone was generated by a function generator (FG-7002C, EZ digital) at 81 dB SPL. The sine tone was played for 7 days to Human Bone Marrow Mesenchymal Stem Cells (hBM-MSCs). The sound treatment induced neural differentiation, and MAP2 and NF-L protein levels significantly increased by more than 150% without altering the cell viability compared to the control group. Phosphorylation levels of Pyk2, ERK, Akt, and CREB were also tested at 10, 45, 90, and 180 min post-stimulation. Pyk2 level spiked (>200%) at 45 min and decreased after 90 min. The ERK level was increased (>150%) from 45 min and stayed until 180 min ($p<.05$). The CREB level was increased (>200%) from 90 min but slightly decreased (∼150%) at 180 min ($p<.05$). The Akt level showed no difference. The Pyk2 level was rechecked at a different SPL (100 dB SPL), but did not show any effect, which suggests a possible SPL dependency.

Table 1. List of Included Studies (in order of Publication Year).

Publication year	First author	Title	Source
2010	Shaobin et al.	A pilot study of the effect of audible sound on the growth of Escherichia coli	*Colloids and Surfaces B: Biointerfaces*, *78*(2), 367–371
2012	Aggio et al.	Sonic vibration affects the metabolism of yeast cells growing in liquid culture: a metabolomic study	*Metabolomics*, *8*(4), 670–678
2016	Mohammed et al.	The effects of acoustic vibration on fibroblast cell migration	*Materials Science and Engineering: C*(69), 1256–1262
2016	Choi et al.	Sound waves induce neural differentiation of Human Bone Marrow-Derived Mesenchymal Stem Cells via ryanodine receptor-induced calcium release and Pyk2 activation	*Applied Biochemistry and Biotechnology*, *180*(4), 682–694
2016	Lestard & Capella	Exposure to music alters cell viability and cell motility of human nonauditory cells in culture	*Evidence-Based Complementary and Alternative Medicine*, *2016*: 6849473, 1–7
2016	Shah et al.	Sound stimulation can influence microbial growth and production of certain key metabolites	*Journal of Microbiology, Biotechnology and Food Sciences*, *9*(5), 330–334
2017	Sarvaiya & Kothari	Audible sound in form of music can influence microbial growth, metabolism and antibiotic susceptibility	*Journal of Applied Biotechnology & Bioengineering*, *2*(6), 212–219
2017	Ventura et al.	Cell melodies: when sound speaks to stem cells	*CellR4*, *5*(2): e2331
2018	Mehrafsar et al.	Effect of exposure to Quran recitation on cell viability, cell migration, and BCL2L12 gene expression of human prostate Adenocarcinoma cell line in culture	*Health, Spirituality and Medical Ethics*, *5*(4), 46–52
2018	Algieri et al.	Effects of music playing on biological molecules	*MATEC Web of Conferences*, *210*: 05006
2018	Kumeta et al.	Cell type-specific suppression of mechanosensitive genes by audible sound stimulation	*PLOS ONE*, *13*(1): e0188764
2018	Kothari et al.	Influence of a mono-frequency sound on bacteria can be a function of the sound-level	*Indian Journal of Science and Technology*, *11*(4), 1–9

In Mohammed et al. (2016), a thin mylar speaker (⌀ = 4.5 cm, 0.2 W) was placed directly underneath the cell culture dish (⌀ = 3.5 cm). A sine tone with different frequencies (100, 200, 400, 800, 1600 Hz) was generated using an Arduino microcontroller. This induced vibrations in the cell culture dish at a particular displacement amplitude (9, 5, 2, <1, <1 µm, respectively). The amplitude was measured by using a laser vibrometer (Polytech Ltd.). The dish was resting on the speaker. Different frequencies were applied for 5 min each time to two different cell types: human lung fibroblast cells (LL24) and mouse fibroblast cells (L929), placed in separate cell culture dishes. The lowest frequency (100 Hz) was found to increase the migration distance of both cell types by 6% (p<.05) over 4 h, without altering the cell viability. The migration distance decreased as the frequencies of the sine tone became higher.

In Kothari et al. (2018), a speaker (Minix soundbar) was placed 15 cm away next to the cell tubes inside a glass box-shaped chamber (25 (l) × 25 (w) × 15 (h) cm). A 300 Hz sine tone was played from a computer software NCH Tone Generator at five different SPLs (70, 76.5, 83, 87.5, 89.5 dB SPL) for 48 h each time. The stimulated cells were bacteria of type *Chromobacterium violaceum*. The noise level was below 40 dB SPL in the control chamber. The result showed an SPL dependency. It is important to note that the SPL difference of less than 13 dB SPL showed no significant alterations in the growth of cells. The cell growth was upregulated the most by 15.02% (p<.001) at 70 dB SPL. Quorum Sensing regulated pigment production was affected the most which increased by 59.63% at 89.5 dB SPL (p<.01).

In Kumeta et al. (2018), a 4-inch active speaker (Fostex 6301NB) was placed above (25 cm away) the cell culture dish inside a cell incubator. A 440 Hz sine tone (triangle, square tones, and white noise were tested too) was generated at different SPLs (76, 82, 88, 94 dB SPL) using NCH Tone Generator on a computer. Each tone was played for 2 h. The background noise inside the incubator was measured to be around 65 dB SPL. The results showed a dependency on the type of waveform used on mice cells (ST2). The sine tones downregulated the gene (Ptgs2, CTGF, TNC) expression level between 60% and 80% (p<.01). The triangle tones downregulated only one of the genes (TNC), and the square tones and white noise did not show any significant effect compared to the sine tones. Different frequencies (55, 110, 4000 Hz) were also tested for the sine tone condition, but the effect was similar to that of 440 Hz. The sound level of 94 dB SPL showed a significant impact, while tones with other SPL showed little effect. Another important finding in this study was the temperature change in the cell culture media liquid. When sound was played at 94 dB SPL, it caused the temperature in the media to increase by 0.4 °C. A noteworthy assumption was that the sound energy

Table 2. Simple Sound Stimulus Studies (in order of Publication Year).

Author (Year)	Cell type	Sound type	Sound frequency (Hz)	Sound pressure level (dB SPL) /Amplitude (μm)	Duration	Key outcome
Shaobin et al. (2010)	Bacteria cell (*Escherichia coli*)	Sine tone	1,000	90 dB SPL	60 (stimulation) + 180 (break) × 6 times	Cell colony forming upregulated to 141.6%
			5,000			Cell colony forming upregulated to 130%
			10,000			Cell colony forming upregulated to 131.1%
Aggio et al. (2012)	Yeast cell (*Saccharomyces cerevisiae*)	Sine tone	100	92 dB SPL	14 h	Growth rate 8.7% higher (p<.005)[d], biomass production up to 14% lower (p<.05)
			10,000	89 dB SPL		Growth rate 11.5% higher (p<.005)[d], biomass production up to 14% lower (p<.05)
Choi et al. (2016)	Human cell (Human BM-Mesenchymal Stem Cells (hBM-MSCs))	Sine tone	1,000	81 dB SPL[c]	168 h	Induced neural differentiation (increased Pyk2 and CREB level p<.05)
Mohammed et al. (2016)	Human cell (LL24; lung fibroblast)	Sine tone	100	9 μm[a]	5 min	Increased cell migration by 6% (p<.05), diminishes after 2~3 h
			200	5 μm[a]		Decreased cell migration compared to 100 Hz (no significant change)
			400	2 μm[a]		Decreased cell migration compared to 400 Hz (no significant change)
			800	<1 μm[a]		No change in cell migration
			1,600	<1 μm[a]		Decrease in cell migration by 50% (p<.05)
Kothari et al. (2018)	Bacteria cell (*Chromobacterium violaceum*)	Sine tone	300	70, 89.5 dB SPL	14 h	70 dB SPL: Cell growth of C. Violaceum by 15.02% (p<.001); 89.5 dB SPL: QS-regulated pigment production increased by 59.63%
Kumeta et al. (2018)	Mouse cell (ST2; bone marrow)	Sine tone	55	76, 82, 88, 94 dB SPL	120 min	Similar effect to 440 Hz in the same study
			110			Similar effect to 440 Hz in the same study
			4,000			Similar effect to 440 Hz in the same study
		Sine, Triangle, Square tones[b]	440			Sine: Ptgs2, CTGF, TNC expression downregulated to 60%–80% (p<.01), Triangle: downregulated only TNC, Square: no significant effect

[a]The amplitude was measured by a laser vibrometer.
[b]White noise was also tested but the effect was insignificant.
[c]The sound was also played at 100 dB SPL, but did not show any effect
[d]It caused less biomass production up to 14% (p<.05).

would be reflected at the lid of the petri dish by more than 10 dB SPL. The transmitted sound pressure into the liquid-based media eventually was calculated to be approximately 10.4 mPa.

Complex Sound Stimulation on Cell Cultures

Here we will look at studies that have used different types of complex sound. This includes the use of music (different styles and genres) or speech (human voice) to stimulate the cell cultures in various experimental settings. An overview of the findings can be found in Table 3.

In Aggio et al. (2012), the setup is described in the previous section. The description of the music is limited; they describe it as “broad-band music” sampled at a bit rate of 320 kbps. The growth rate showed an increase by 12.4% (p<.005) when the music was played to the cell culture, which caused a decrease in biomass production by 14% (p<.05). This observation was similar when the sine tone was played to the cell culture in the same study.

Table 3. Complex Sound Stimuli Studies (in order of Publication Year).

Author (Year)	Cell type	Sound type	Sound frequency range (Hz)	Sound pressure level (dB SPL)	Duration	Key outcome
Aggio et al. (2012)	Yeast cell (*Saccharomyces cerevisiae*)	Broad-band music	-	80–92 dBA	14 h	Growth rate 12.4% higher (p<.005)[a]
Lestard and Capella (2016)	Human cancer cell (MCF-7 & MDA-MB-231)	Mozart's Sonata for Two Pianos in D Major, KV. 488	-	70–100 dB SPL	30 min	Caused apoptosis in MDA-MD-231, not in MCF-7 (p<.05)
		Beethoven's 5h symphony, 1st movement	-			Decreased the number of living cells 48 h post-stimulation, inhibited the migration of MDA-MD-231 (p<.05)
		Ligeti's Atmospheres	-			Caused apoptosis in both cell lines (p<.05)
Shah et al. (2016)	Prokaryotic & eukaryotic microorganisms	Hindustani classical music (1. *Ahir Bhairav* & 2. *Piloo*)	1. 172–581 2. 86–839	1. 70–90 dB SPL 2. 85–110 dB SPL	-	Growth rate of the microorganisms was changed depending on the cell type (refer to discussion below)
Sarvaiya and Kothari (2017)	Prokaryotic & eukaryotic microorganisms	Indian classical piece (Raag Malhar)	41–645	95–110 dB SPL	-	Increased growth rate and antibiotic susceptibility except Serratia marcescens (p<.05)
Ventura et al. (2017)	Human cell (hASCs)	Percussive music based on African & Latin American traditional rhythms and sounds	70 (fundamental frequency)	~50 dB SPL	-	Different multispectral imaging patterns were generated by stem cells in response to different sound
		Human voice (soft / loud)	100 / 200 (fundamental frequency)	~40 / 30 dB SPL	-	
Algieri et al. (2018)	Biological molecules (tyrosinase)	Original music composition (Reminiscenza)	523.25, 587.33, 659.26	~75 dB SPL	-	Increased production by 30% (p<.05)
		Original music composition (Pioggia)	261.63, 293.66, 523.25, 622.25, 698.46, 830.61	~60.5, 75.5, 81 dB SPL	-	Decreased production by 50, 60, 80% at 60.5, 75,5, 81 dBA respectively (p<.05)
Mehrafsar and Mokhtari (2018)	Human cancer cell (PC-3)	Quran recitation (Surah Al-Fatina, voice by S.A. Basit)	-	90 dB SPL	120 min	91.8% (p<.05) of cells survived, migration rate decreased to 95.4% (p<.05), mRNA level of BCL2L12 decreased by a factor of 0.48 (p<.001)

[a]It caused less biomass production up to 14% (p<.05).

In Lestard and Capella (2016), a 60 W coaxial speaker was installed at the top of the acoustically treated (with cork and foam) incubator. The first movement from Mozart's Sonata for Two Pianos in D Major, KV. 488, the first movement from Beethoven's 5th symphony, and Ligeti's *Atmospheres* were played to the cells. These excerpts were played at around 70–100 dB SPL for 30 min each to human breast cancer cells (MCF-7 and MDA-MB-231). Mozart's piece did not alter the viability of MCF-7 but caused cell death (apoptosis) in MDA-MD-231 (p<.05). The Beethoven and Ligeti pieces decreased the number of living cells 48 h post-stimulation and inhibited the migration of MDA-MD-231 (p<.05). The Ligeti piece caused significant cell apoptosis in MCF-7 (p<.05).

In Shah et al. (2016), a speaker was placed next to (15 cm away) the cell tubes inside a glass chamber (22.5 (l) × 22.5 (w) × 12.5 (h) cm). The SPL was measured with an SPL meter (ACD Machine Control Company) that was positioned 15 cm away from the speaker. Two pieces of

music (*Ahir Bhairav* and *Piloo*) were played at 70–90 dB SPL and 85–110 dB SPL, respectively, to six different microorganisms (prokaryotic and eukaryotic). The recordings were from a CD album named *Call of the Valley* (Saregama Indian Ltd.). The fundamental frequency ranges of the two pieces were 172–581 Hz (*Ahir Bhairav*) and 86–839 Hz (*Piloo*). After playing *Ahir Bhairav*, the growth rate of *Serratia marcescens* (−4.32%, $p<.01$), *Chromobacterium violaceum* (4%, $p<.05$), *Xanthomonas campestris* (6.66%, $p<.05$), *Saccharomyces cerevisiae* (8.94%, $p<.01$), *Lactobacillus plantarum* (6.14%, $p<.01$), and *Bacillus parabrevis* (18.75%, $p<.01$) were changed compared to the control. After playing *Piloo*, the growth rate of *Serratia marcescens* (−7.46%, $p<.01$), *Chromobacterium violaceum* (−24.19%, $p<.01$), *Xanthomonas campestris* (9.32%, $p<.05$), *Saccharomyces cerevisiae* (4.68%, $p<.01$), *Lactobacillus plantarum* (3.41%, $p<.01$), and *Bacillus parabrevis* (18%, $p<.05$) were changed compared to the control.

In Sarvaiya and Kothari (2017), the experimental setup was the same as in Shah et al. (2016). A piece of music (*Raag Malhar*) was played at around 95–110 dB SPL to various microorganisms (prokaryotic & eukaryotic). The fundamental frequency range of the music was between 41 and 645 Hz. All tested cells showed an increase in growth rate, and in antibiotic susceptibility, except *Serratia marcescens* ($p<.05$). Another key finding was the enhanced alcohol tolerance of *Saccharomyces cerevisiae* was significantly enhanced by 33.69% ($p<.001$) after 24 h of incubation when the concentration of ethanol was 5%v/v, 132.94% ($p<.001$) after 24 h of incubation when the concentration of ethanol was 10%v/v, 27.92% ($p<.01$) after 48 h of incubation when the concentration of ethanol was 12.5%v/v, and 1200% ($p<.001$) after 120 h of incubation when the concentration of ethanol was 15%v/v.

In Ventura et al. (2017), the cell culture was placed 2 m in front of a live performance. Percussive music based on traditional rhythms and sounds from Africa and Latin America and an actor's voice were played for 45 and 30 min, respectively, to Human Adipose Stem Cells (hASCs). The tempo of the percussive music was taken from the performer's own heartbeat, and the actor's voice was performed with two different intensities (soft and loud). The most dominant frequency for the percussive music was around 70 Hz at ∼50 dB SPL, and for the actor's voice was around 100 Hz at ∼ 30 dB SPL (soft voice) and 200 Hz at ∼30 dB SPL (loud voice). The sound analysis was done through Praat, a software package developed for speech analysis. The cell culture was observed in real time under the Multi-Spectral Imaging (MSI) system (Nikon Eclipse TS 100). The MSI analysis showed spectral changes of cell imaging with specific vibrational signatures in relation to the performances.

In Algieri et al. (2018), a set of 3 W internal speakers (LENOVO E93z) was placed underneath (10 cm away) the reactor vessel on a magnetic plate. The SPL measurement was done with a FUSION −1 dB meter and analyzed using dB Trait. Two distinctively composed pieces of music, *Reminiscenza* (constant rhythm and melody) and *Pioggia* (irregular rhythm with more variable devices such as irregular tempo changes, and melody) were played to biological molecules (tyrosinase). These were placed in a reactor vessel with a stirring mechanism and a constant temperature of 30 °C. In *Reminiscenza*, only C5 (523.25 Hz), D5 (587.33 Hz), and E5 (659.26 Hz) musical notes were used and played at ∼75 dBA. In *Pioggia*, C4 (261.63 Hz), D4 (293.66 Hz), C5 (523.25 Hz), D#5/Eb5 (622.25 Hz), F5 (698.46 Hz), and G#5/Ab5 (830.61 Hz) were used, and played at different SPL (60.5, 75.5, and 81 dBA). As one of the key findings, the 1-DOPA production rate was reported. Playing *Reminiscenza* resulted in a significant increase in the 1-DOPA production, by 30% more compared to the control group. Playing *Pioggia* resulted in decreased the 1-DOPA production by 50% at 60.5 dBA, 60% at 75.5 dBA, and 80% at 81 dBA ($p<.05$).

In Mehrafsar and Mokhtari (2018), four speakers were placed around the cell culture plate. A voice recording (*Surah Al-Fatina,* voice by *Sheikh Abdul Basit*) was played at 90 dB SPL for 2 h to the human cancer cell line (PC-3; Human Prostate Adenocarcinoma Cells). The cell viability, migration, and BCL2L12 gene expression level were reported. After the recorded audio was played to the cells, it was reported that 91.8% ($p<.05$) of PC-3 cells

Table 4. The List of Proposed Cellular Sensing Mechanisms or Type of Forces Induced by Sound Waves.

First author/date	Proposed cellular sensing mechanisms/ type of forces induced by sound waves at the cellular level
Shaobin et al. (2010)	• Induced oscillation of cells • Induced motion of internal fluid (inside the cells) • Deformation of plasma membrane • Increased membrane fluidity
Choi et al. (2016)	• Mechanical stress on the membrane of the cells
Lestard and Capella (2016)	• Mechanical stress on the cells
Shah et al. (2016)	• Induced vibration within the cell medium (liquid) • Different pressure distribution in membranes ("pressure profile of membrane") of different cell types
Sarvaiya and Kothari (2017)	• Induced vibration within the cell media (liquid)
Ventura et al. (2017)	• Induced oscillation of the cells
Mehrafsar and Mokhtari, (2018)	• Mechanical stress on the cells
Algieri et al. (2018)	• Induced oscillation of the cells
Kumeta et al. (2018)	• Induced oscillation of the cells: leading to molecular and cytoskeleton sensing

survived, the migration level decreased to 95.4% ($p<.05$), and mRNA level of BCL2L12 decreased by a factor of 0.48 ($p<.001$).

Possible Cell Sensing Mechanisms of Sound

Many of the reviewed studies have proposed possible ways that cells sense mechanical cues due to sound stimulation. Some of the speculations reported are compared in Table 4.

In the subsequent sections, we will discuss some of the central issues from the selected studies.

Discussion

Many different types of sound (simple and complex), with an extensive range of audio frequency and SPL have been tested. The results reported for both simple and complex sound studies are promisingly beneficial. Some of the important observations include enhanced cell migration, proliferation, colony formation, and upregulation of certain genes and proteins or decrease in cancer cell migration and inducing apoptosis of cancer cells. However, it is both challenging to find a systematic pattern across studies and to compare the results to test for publication bias because the studies used different statistical analysis and effect sizes, sounds, experimental setups, sound measurement methods, and observation strategies. Below, we synthesize and discuss the results while considering the practical aspects of the studies (e.g., experimental setups and measurement methods) and the physics of sound.

Experimental Setup

Many of the reviewed studies were based on a speaker position above (Figure 2; scenarios 1 and 2, position a.), underneath (Figure 2; scenarios 1 and 2, position b.) or outside an incubator or specially constructed chamber for the cell cultures (Figure 2; scenario 1, position a.). The distances between the speaker and petri dish varied, ranging from ~0 (directly underneath the dish) to 200 cm. These speaker placements claimed to have resulted in cellular responses regardless of whether the speaker was placed outside or inside a specially constructed chamber or cell incubator. Whether the observed biological alterations are a direct effect of sound pressure waves is doubtful in some cases such as speaker position c in scenario 1 in Figure 2. As discussed earlier, there are several physical factors that contribute to the transmission loss of sound energy. This was discussed by one of the studies where the polystyrene cover of petri dishes and significant impedance mismatch between water and air were taken into consideration to estimate the transmission loss (Kumeta et al., 2018). Theoretically, the amount of acoustic energy that reaches the cells would be insignificant compared to the incident energy as elaborated above.

It would be of value to perform a systematic experiment of how different positioning of the sound source (the speaker) results in various types of mechanical force in the cell culture media. For example, whether a speaker is coupled (in direct contact) with the petri dish. Several of the reviewed studies have not clearly stated how the experimental environment was acoustically treated. Based on its physical properties, sonic vibrations may interact with the testing environment. Ideally, sound experiments should be performed in an acoustically controlled environment. For example, an anechoic chamber is designed to imitate the free field conditions (i.e., no reflected sound waves), and to allow no or minimum external sound to enter the chamber. Using such an environment can greatly improve the accuracy of acoustic measurements (González et al., 2018). Additionally, for practical reasons, speakers should ideally be placed inside or facing directly into an acoustically treated chamber. Another reason would be to prevent hearing damage of the researchers. For example, a continuous sine tone at a loudness level of 70 dB SPL or higher can cause temporary or even permanent hearing damage (Huber, 2010). Although, a full anechoic

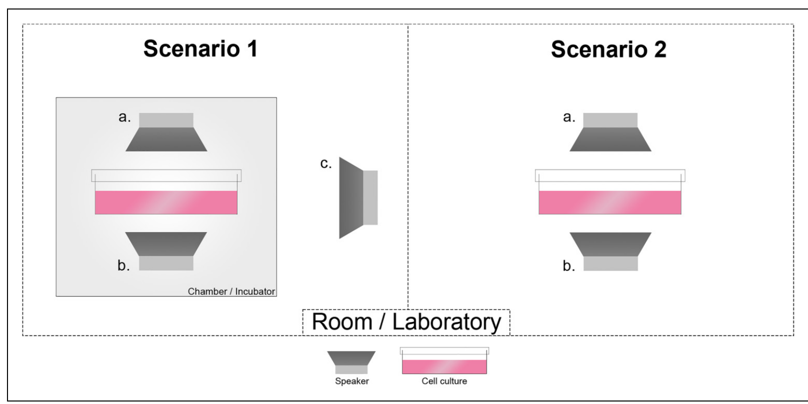

Figure 2. Different positions (scenarios) of the speaker used in the experiments.

chamber would not be feasible considering the cost and size, it would still be useful to try to reduce the acoustic impact of the environmental "noise" by using an acoustic damping device. In that way, it becomes more possible to measure and characterize how the sound behaves in an environment. This could also be used to address potential obstacles during experiments (e.g., amplitude distortion at specific frequencies, standing waves, and phase cancelation) and increase the chance to infer how different acoustic conditions are related to observed cellular responses.

Sound Features

We will look at four basic sound features here: frequency (Hz), sound pressure level (dB SPL), waveform (simple & complex), and rhythm. The overview of the studies is illustrated in Figure 3.

Frequencies. In the studies where single-frequency sound was used, a single frequency selected in the range of 55–10000 Hz were explored on bacteria cells (*Escherichia coli*, *Chromobacterium violaceum, Serratia marcescens*), mouse cells (ST2), and human cells (hBM-MSCs, LL24). In the studies where the more complex sounds were used, musical pieces or human voices over a wide frequency range were explored on a biological molecule (tyrosinase), microorganisms (various prokaryotic & eukaryotic), bacteria cells (*Escherichia coli*), human cancer cells (MCF-7, MDA-MD-231), and human cells (hASCs).

To summarize, bacteria and yeast cells showed an effect of sound across a wide range of frequencies: cell colony-forming and growth rate increase using 100, 300, 1,000, 5,000, and 10,000 Hz (Aggio et al., 2012; Choi et al., 2016; Lestard & Capella, 2016). On the other hand, mammal cells showed a more selective and frequency-dependent effect. For example, a lower frequency (100 Hz) induced cell migration increase in human cells, but higher frequencies (above 1600 Hz) induced the opposite effect on cell migration (Mohammed et al., 2016). Also, a sine tone of 440 Hz induced gene (Ptgs2, CTGF, TNC) expression increase in mouse cells (ST2), while 55, 110, and 4,000 Hz showed a less or similar effect (Kumeta et al., 2018).

In the complex sound studies, the styles of music and human voice employed varied widely (Table 5). The most present frequencies of the music and voices tested stayed between 40 and 840 Hz. However, it is most likely that other frequency content, especially overtones, varied between different types of music or speech style due to distinctive *timbre* differences between instruments and human voices. Since complex sounds typically consist of many frequencies, it is difficult to know if a particular frequency had an impact on the cellular responses. Regardless, a notable

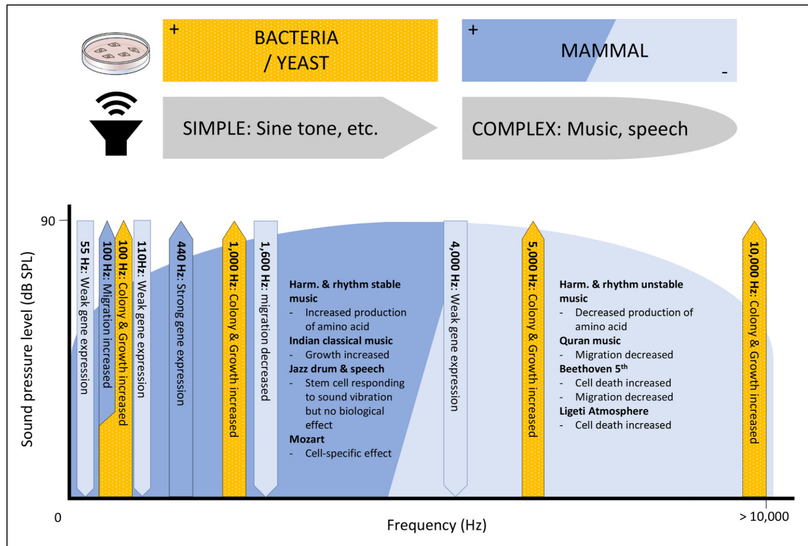

Figure 3. A simplified schematic diagram of the outcome of the studies. A wide range of sound frequency was tested (approximately between 20 and 10,000 Hz) with the sound level being around 90 dB SPL. The studies are divided into bacteria/yeast and mammal cells studies. The studies are further categorized into biological outcome as positive (upregulation) and negative (downregulation), and types of sound as simple (sine tone) and complex (music and speech). Since each experiment had a specific cell line and a setup, it was not possible to draw a full conclusion. However, the diagram provides an overview and some recognizable patterns in the result. Concerning these specific sets of outcomes, mammal cells showed more frequency dependency than bacteria cells. A group of studies of bacteria/yeast cells reported a general increase of colony-forming and growth rate over a wide range of frequencies, whereas, in mammal cell studies, the effects of the sound depended more on which frequency was used.

outcome was where two musical compositions of contrasting characteristics in terms of melody and rhythm (regular/constant vs irregular/spontaneous) showed contrasting results in l-DOPA production rate in *tyrosinase*. A musical composition with regular characteristics, *Reminiscenza*, resulted in l-DOPA production increase. This piece of music is described, by Algieri et al. (2018), as having stability in terms of harmony and rhythm. The entire piece is based on a thetic rhythm and centered around 523.25 Hz (musical note: C5), 587.33 Hz (D5), and 659.26 Hz (E5). Another piece of music that had a contrasting characteristic, *Pioggia*, resulted in l-DOPA production decrease. Unlike the first piece, *Pioggia* is described as more unstable and based on agogic accents and rubato, and frequencies varied more using 261.63 Hz (C4), 293.66 Hz (D4), 523.25 Hz (C5), 622.25 Hz (D#5/Eb5), 698.46 Hz (F5), and 830.61 Hz (G#5/Ab5) (Algieri et al., 2018). This indicates a possible role of the degree of pitch (frequency) and rhythm in the produced outcome.

Table 5. The List of Music and Voice Styles From the Selected Studies.

First author	Type/style	Additional information
Lestard and Capella (2016)	Western classical music	Mozart, Beethoven, Ligeti
Sarvaiya and Kothari (2017); Shah et al. (2016)	Hindustani classical music	*Ahir Bhairav, Piloo, Raag Malhar*
Ventura et al. (2017)	African and Latin American	Percussive music: Milford Graves
	Acting voice	Whisper and clang
Algieri et al. (2018)	Contemporary original music	*Reminiscenza* and *Pioggia*
Mehrafsar and Mokhtari (2018)	Quran recitation	*Surah Al-Fatina*

Sound Pressure Level. In the investigated studies, although the sound pressure level range used varied extensively, from 40 to 110 dB SPL, the majority of the studies used the range between 75 and 95 dB SPL. The range centered around 90 dB SPL, which is about 632 mPa. This pressure level is small compared to the periodic blood flow pressure, for example, which can typically be around 120/80 mmHg (approximately 10–16 kPa) (Zhou et al., 2017). The transmitted sound energy in the cell media (liquid) from the air is estimated to be even smaller: approximately 29.67 mPa (when the temperature is 20 °C, $R \approx 0.9988$, transmitted sound energy from the air to water is $1 - R \approx 0.0022$ when there is no absorption) (Kumeta et al., 2018). This is a insignificant mechanical stress, but the cells have mechanisms that can sense such small forces and changes involving cytoplasmic molecules α-catenin, for example (Maki et al., 2016).

It should be noted that some of the SPLs specified in the literature are incomparable. This is because some of the reports were unclear about how the actual sound output was measured. Additionally, some of the commercially available SPL measurement devices typically come with filters that imitate the human hearing characteristics (less sensitive at very low and high frequencies within the audible frequency range), such as an A-weighting filter. There are other weightings, as illustrated in Figure 4

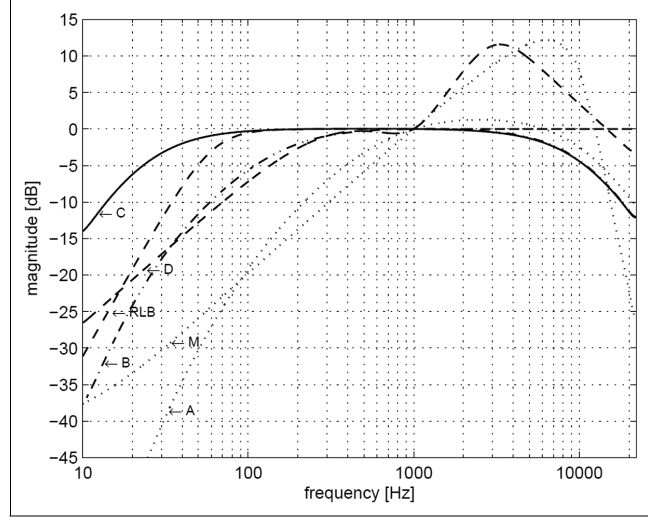

Figure 4. Frequency response of the A, B, C, D, M, and RLB frequency weighting filters. All the curves are aligned in level so that their magnitude response at 1 kHz is 0 dB. The figure is reproduced with permission (Skovenborg & Nielsen, 2004).

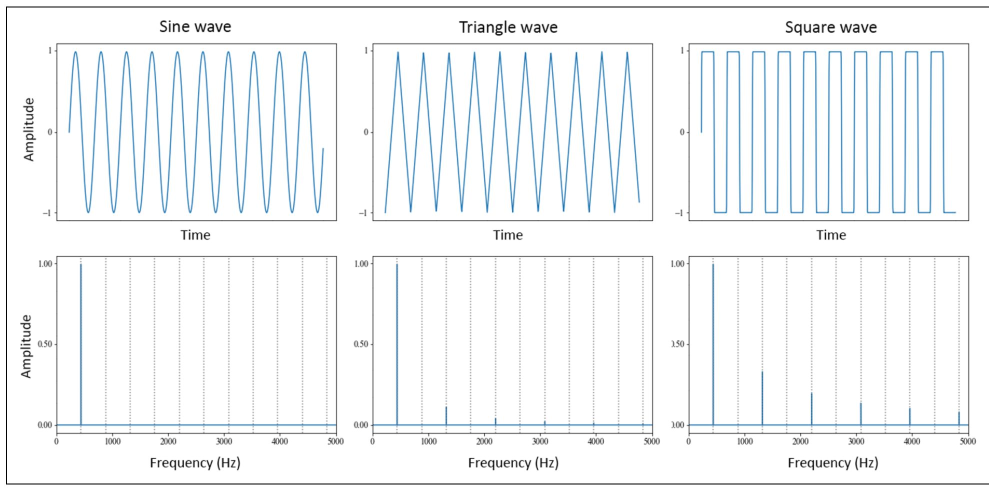

Figure 5. Comparisons between 440 Hz sine, triangle, and square waveforms (top) and Fourier Transforms (below).

(Skovenborg & Nielsen, 2004), designed for different applications. This is a small piece of information that may not be considered necessary, but it should be stated in the report, as a specific weighting filter readout can undermine a particular frequency range as shown.

Waveform. As pointed out earlier, the majority of the simple sound studies employed sine tones. There was only one study that compared different waveforms (triangle and square) and white noise. Interestingly, the different waveforms yielded contrasting outcomes in cellular responses (Kumeta et al., 2018). However, the underlying mechanism is not well understood. Sine waves do not have any harmonics (overtones), only the fundamental frequency. Triangle and square waves, on the other hand, contain odd harmonics of the fundamental frequency. The higher harmonics of the triangle wave get weaker faster than those of a square wave, as shown in Figure 5. When the amplitude and the frequency are the same, there is more sound energy in square waves and stronger harmonics than in sine and triangle waveforms. Although there is a need for further investigation, according to the differences we explore, a possible explanation of different outcomes between sine, triangle, square, and white noise could be the strength of harmonics present in the sound material.

Rhythms. Rhythm is one of the fundamental properties of music and is also an essential feature of biological life. The "synchronization of multiple rhythms" is vital in living organisms (Muehsam & Ventura, 2014), and rhythmicity as a parameter is underexplored. The human body is full of different rhythms, such as heart rate and breathing rhythms. If certain rhythms in the human body become irregular, it can be an indication of more severe health problems, for example, abnormal respiration rhythms (Whited & Graham, 2020).

In the investigated simple sound studies, there were no rhythmic features in the sound. On the other hand, all the complex sound studies had an aspect of rhythm. The styles of rhythms varied between Western classical music, Hindustani classical music, and African and Latin-American traditional music. For illustration, Mozart's Sonata for Two Pianos in D Major, KV. 488 shows more regular and steady rhythmic patterns than Ligeti's *Atmospheres* (Figure 6a and b). There was only one study in which the aspect of rhythm was deliberately tested and reported, and where regular/constant and irregular/inconstant rhythms were compared in terms of cellular responses (Algieri et al., 2018). As discussed previously, the results showed that the musical piece with regular rhythm produced an increase in the production of l-DOPA compared to the control group, whereas the piece with irregular rhythms resulted in a decrease, although other factors in the pieces of music could have contributed to the difference.

Other possible premises can be reflected upon when it comes to the rhythmic stimulation of *in vitro* cells. For instance, the regulation of intake of Ca^{2+} is a crucial factor for insulin secretion β-cells (Yang & Berggren, 2006), which is regulated by Ca^{2+} channels located in the cell membrane (Larsson-Nyrén et al., 2007). The Ca^{2+} intake of β-cells is known to be rhythmic (Idevall-Hagren & Tengholm, 2020), and the mechanical force can regulate the Ca^{2+} intake (Matthews et al., 2010; Yang et al., 2015). Therefore, it would be interesting to explore the question of whether a rhythmic mechanical stimulation can "rescue" irregular or un-synchroneity Ca^{2+} oscillation (Li et al., 2014; Yang et al., 2015) in unhealthy β-cells. Taken together, rhythmicity is a possible new avenue of sound stimulation of cells to be investigated further.

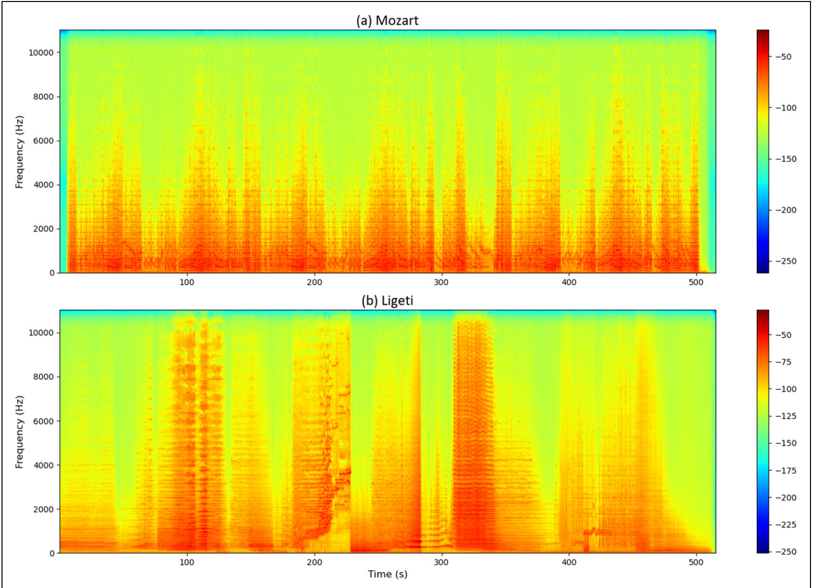

Figure 6. Spectrograms of (a) the first movement of Mozart's Sonata for Two Pianos in D Major, KV. 488 and (b) Ligeti's Atmospheres.

Cell Sensing Mechanisms of Sound

There have been some reports on cellular changes after a certain period of exposure to sound waves. Although several possible mechanisms have been suggested (Table 4), the studies in the current review unanimously acknowledged the lack of understanding of the mechanism by which cells are affected by sound waves. One of the aspects that needs more attention is a possible shear force generated by the flow of the fluid (cell culture media) within the petri dish when sound is played to the cell culture. Shah et al. (2016) and Sarvaiya et al. (2017) partly referred to a possible effect of shear force by suggesting that sound induces vibration within the cell culture media. Nevertheless, it is not clear whether fluid flow has any effect on the reported outcomes. Such mechanical oscillations could potentially have an impact on the cellular responses in addition to the sound waves (Kanie et al., 2019).

Conclusion

There appears to be promising effects of using audible sound on microorganisms and mammalian cells. However, the correlations are complex, and the exact cause of cell responses to audible sound stimulation is still largely underexplored. We have also found that there are considerable differences in experiment setups and their acoustic environment. This makes it difficult to understand how the stimulation happened. In future research, therefore, we see the need for:

- **Controlled sonic environments**: An acoustically treated environment would make it easier to control the sound. This would reduce the level of external noise and avoid unwanted or uncontrolled reinforcement or cancelation of frequencies. What is being applied to the cells should ideally be the sound emitted directly from the sound source. The use of an anechoic chamber or appropriate acoustic treatment of the experimental environment is recommended with minimum acoustic barriers (e.g., incubator walls) between the sound source and the cell cultures.
- **Consistent measurement methods**: Due to the possible (sonic) influence of the environment, it is necessary to measure and analyze the environment with appropriate equipment (e.g., a flat frequency response calibration microphone). This would serve as a control, and an appropriate SPL measurement method with a weighting filter should be used to estimate what the cells are stimulated with.
- **Broad and systematic synthesis of sound**: A more comprehensive range of controlled parameters of sound, or possibly music, should be tested. All the simple sounds used in the literature so far have been of a continuous nature. Considering the complexity of the ECM (environment) that the cells live in, learning the influence of pulsating or rhythmic stimulation on the cell cultures could also be valuable.

Many other possible non-sound related factors can alter the functionality of cells under examination (Goetzke et al.,

2018). Therefore, as to recommendations for future research in this multidimensional and uprising field, it is crucial to have the sound waves well under control and elucidate the practical issues. High priority should be given to investigating the optimal acoustic environment for cell stimulation that takes cell viability (e.g., temperature) into consideration. The focus should also be put on standardizing methods to measure and analyze the characteristics of the experimental sound output and the background noise. Finally, we suggest replication studies of the findings from the literature under the above-mentioned controlled conditions.

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Peer Review

Silas Villas-Boas, University of Auckland, School of Biological Sciences.
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Paper II

A trio of biological rhythms and their relevance in rhythmic mechanical stimulation of cell cultures

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A trio of biological rhythms and their relevance in rhythmic mechanical stimulation of cell cultures

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The primary aim of this article is to provide a biological rhythm model based on previous theoretical and experimental findings to promote more comprehensive studies of rhythmic mechanical stimulation of cell cultures, which relates to tissue engineering and regenerative medicine fields. Through an interdisciplinary approach where different standpoints from biology and musicology are combined, we explore some of the core rhythmic features of biological and cellular rhythmic processes and present them as a trio model that aims to afford a basic but fundamental understanding of the connections between various biological rhythms. It is vital to highlight such links since rhythmic mechanical stimulation and its effect on cell cultures are vastly underexplored even though the cellular response to mechanical stimuli (mechanotransduction) has been studied widely and relevant experimental evidence suggests mechanotransduction processes are rhythmic.

KEYWORDS

biological rhythms, cellular rhythms, rhythmic mechanical stimulation, cell cultures, tissue engineering, regenerative medicine

Introduction

Rhythm is one of the most basic and important elements in music. It usually has a repetitive structure typical of rhythmic signals but is also characterized by small and large deviations from that structure. Accordingly, we think of rhythms as ordered patterns in time. The importance of rhythm in music is comparable with that of rhythm in biological systems: rhythm in music is not trivial but one of the essential devices for musical expressions and an element that makes music "alive," and likewise, rhythm in biological systems is not only an observable phenomenon but necessary for sustaining life. As fundamental biological phenomena (Haken and Koepchen, 1991), rhythmic biological processes are related to the tendency to stay in balance between chaos and order (i.e., homeostasis; Crutchfield, 2003; Gnocchi and Bruscalupi, 2017). We contend that this rhythmic "balancing act" of homeostasis is one of the key biological elements that is insufficiently accentuated and overlooked in the research area of mechanical stimulation

of cell cultures in relation to tissue engineering and regenerative medicine fields, in which providing and mimicking a dynamic *in vivo* environment for *in vitro* cell culture models is an important question.

One of the major developments in tissue engineering has been related to micro-scale structural engineering. For example, spatial variations and their effects on cell cultures have been studied extensively by using various types of technologies such as 3D culturing systems, bioprinting, and organ-on-chip designs (Kim and Hayward, 2012; Lee and Cho, 2016; Jensen and Teng, 2020; Low et al., 2021). The main advantages provided by the intricate structural designs include growing cells in various patterns and shapes and on different material stiffness (e.g., gel or PDMS), which create specific types and varying degrees of mechanical restrictions and forces on the cell cultures. As a result of such improvements, along with recent developments in stem cell technologies, it is now possible to generate organoids and mini tissues that represent the functional characteristics of the organ from which the stem cells were derived (Kratochvil et al., 2019). Optimization of the structural environment of cell culture systems is actively being pursued to advance the development of personalized medicine and drug screening (Kim et al., 2020). However, as biological processes occur spatiotemporally (Grace and Hütt, 2015), what should be as critical as optimal mechanical stimulation by structural cues (i.e., ordered patterns in space) is the optimization of temporal patterns of the mechanical stimulation (i.e., ordered patterns in time). Rhythmic stimulations have been explored, but only in a small number of areas, such as microfluidic systems used on blood vessel cells (endothelial cells; Novo et al., 2016; Yeom et al., 2017; Ortseifen et al., 2020), electrical stimulations used on cardiac cells (cardiomyocytes; Laasmaa et al., 2019), and application of cyclic tensile strain to mimic respiratory motions in lung-on-chip platforms (Huh et al., 2010).

In this article, firstly, we briefly present an overview of biological rhythms in different temporal scales. Secondly, we present a trio biological rhythm model in terms of central rhythm, internal/external rhythm, and reflex/consequential rhythm and discuss how these rhythms are interconnected to regulate homeostasis in a biological system. Thirdly, we explore selected biological and cellular rhythms with critical functions that demonstrate the trio rhythm model in human body organ systems, such as the cardiovascular system (specifically rhythms in relation to blood pressure, blood vessels, and smooth muscle cells) and the digestive system (pancreas, β-cells, and insulin secretion). Lastly, we discuss the potential relevance of the presented trio rhythm model and cellular rhythm examples in the context of rhythmic mechanical stimulation—using various types of experimental apparatuses that can generate controlled mechanical/physical forces such as compression, tension, and shear force directly on the cell cultures—of cell cultures. This article aims to shed light on the rhythmic mechanical stimulation of cell cultures as an area that deserves more consideration in terms of the design of cell culture systems and other cellular experiments in general, but not to provide exhaustive descriptions of biological rhythms or to investigate the origin of biological rhythms, which has been previously discussed in-depth in Haken and Koepchen (1991) and Glass (2001).

Rhythms in different temporal scales

The biological rhythms can be subdivided largely into three levels in terms of temporal scales. Firstly, as shown in Figure 1, ultradian rhythms refer to recurring cycles that are completed more than once per day. For example, molecular and cellular rhythms can occur within seconds to several hours (Goldbeter et al., 2012). Larger structures like cardiovascular and respiratory systems also have rhythms functioning within this scale (Haken and Koepchen, 1991). Secondly, circadian rhythms, which are studied more broadly than the other two rhythms, are recurring cycles completed daily. These rhythms are mainly activated by light and dark patterns. A generally known example would be sleep and wake cycles (Van Someren, 2000; Tähkämö et al., 2019). Thirdly, infradian rhythms refer to recurring cycles which can last longer than a day. The cycles in this time scale can last for months and years. These rhythms include menstrual cycles, human life cycles, and generations of life.

Even though these three rhythm levels are presented as independent levels, there are possible interactions between them (Laje et al., 2018). For example, ultradian rhythms may be harmonics—integer multiples of the fundamental frequency—of 24-h circadian rhythms (Zhu et al., 2018). In some cases, interactions may result in entrainment—the interaction of independent rhythmic systems—between rhythms at different time scales (Kuhlman et al., 2018). This pertains to cellular rhythms *in vivo* that involve changes in melatonin levels and sleep–wake cycles (Gnocchi and Bruscalupi, 2017). For example, one of the main factors that regulate insulin secretion rhythms in β-cells (ultradian and circadian), apart from other factors such as rhythmic inter- and intracellular calcium ion (Ca^{2+}) levels (Daraio et al., 2017), is the circadian melatonin rhythm in the body (Perelis et al., 2016). We will discuss some of these fundamental rhythms and their interrelationship in the following section.

Homeostatic rhythms

A trio of rhythms

We here illustrate biological rhythms as a trio model (Figure 2A). The three rhythms act as stimuli hierarchically, sequentially, and reciprocally depending on the context. The main objective of the trio is to maintain and regulate homeostasis in a given system. The trio model resembles the homeostatic model suggested by Modell et al. (2015). According to their model, there must be (a) sensors or receptors, (b) a control

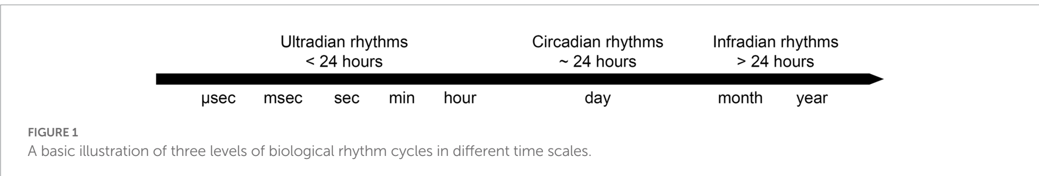

FIGURE 1
A basic illustration of three levels of biological rhythm cycles in different time scales.

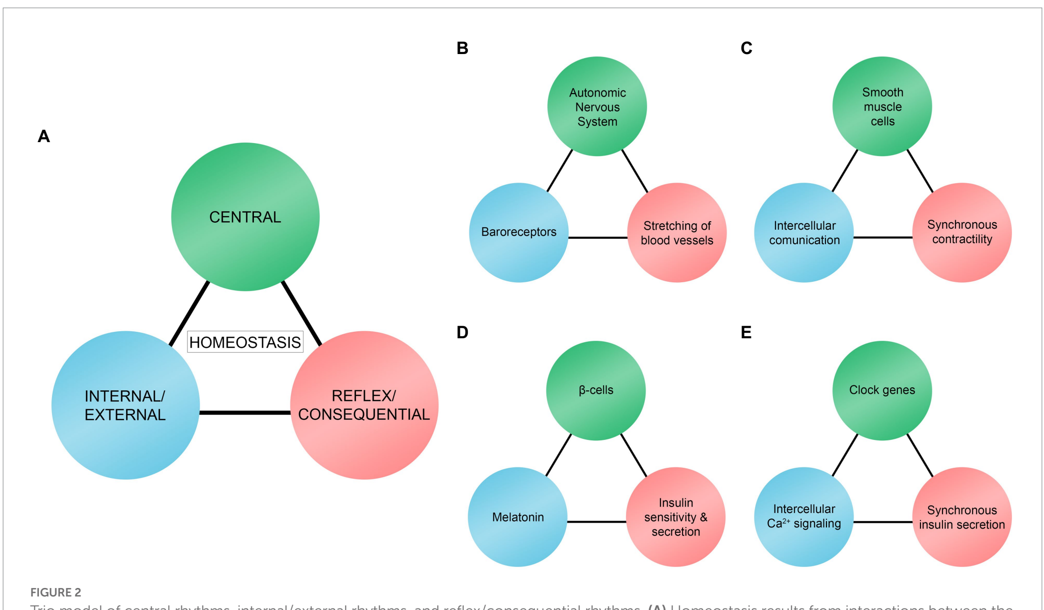

FIGURE 2
Trio model of central rhythms, internal/external rhythms, and reflex/consequential rhythms. **(A)** Homeostasis results from interactions between the trio rhythms. **(B)** Interaction between the autonomic nervous system, baroreceptors, and mechanical stretching of blood vessels to regulate and maintain blood pressure. **(C)** Interaction between smooth muscle cells, intercellular communication, and synchronous contractility of the cells to regulate and maintain rhythmic contractility of smooth muscle cells. **(D)** Interaction between β-cells, melatonin, and insulin sensitivity and secretion as one of the mechanisms to regulate and maintain glucose levels. **(E)** Interaction between clock genes, intracellular Ca^{2+} signaling, and synchronous insulin secretion as one of the mechanisms to regulate and maintain glucose levels.

center for integrating and processing received information, and (c) effectors in homeostatic systems. These are comparable to what we will refer to as central rhythms (control center), internal/external rhythms (sensors or receptors), and reflex/consequential rhythms (effectors). Although our model is similar to the one presented by Modell et al. (2015), there are some differences. In our trio model, internal/external rhythms are not always sensors or receptors. They are more comprehensive and include rhythmic phenomena (e.g., intercellular communication, Ca^{2+} signaling, and melatonin level) that have an essential role in maintaining homeostasis processes in different temporal scales. Moreover, reflex/consequential rhythms pertain more to rhythmic biological responses or phenomena that are different from effectors. Effectors are typically locations or targets that the control center sends signals to, including cells, tissues, and organs (Modell et al., 2015).

In our model, central rhythms are often coming from a specific central location [e.g., Autonomic Nervous System (ANS), smooth muscle cells, and pancreatic β-cells in our examples, which are discussed in the next section] where information is gathered, integrated, and processed. Some typical examples of central rhythms can be the brain, the nervous system, and the nucleus of the cells. Central rhythms are also rhythmic biological phenomena that play a central role in the trio, such as the clock genes regulating synchronous insulin secretion in β-cells.

Central rhythms receive information from and work synchronously with internal/external rhythms. Internal rhythms are endogenous (located or generated within the location of the central rhythms), such as intercellular communication within the group of smooth muscle cells (Figure 2C) and intracellular Ca^{2+} signaling in β-cells (Figure 2E). External rhythms are exogenous (located or generated outside the central rhythms),

such as baroreceptors (Figure 2B) and melatonin (Figure 2D) that are located and generated, respectively, outside the location of the central rhythms. Internal/external rhythms signal the central rhythms of the changes occurring in their immediate environment. Internal/external rhythms are often used as experimental variables that are manipulatable such as levels of melatonin (Pourhanifeh et al., 2020) and Ca^{2+} (Cavieres-Lepe and Ewer, 2021), and electrically activated baroreceptors (Tohyama et al., 2020).

As a result of interactions between central and internal/external rhythms, reflex/consequential rhythms take place. They are either negative or positive deterministic results of the interaction between the first two rhythms. For example, these rhythms are shown through stretching of blood vessels to maintain blood pressure homeostasis (Figure 2B; reflex), enhanced or activation of synchronous smooth muscle cells contractility (Figure 2C; consequential), and increased insulin sensitivity and secretion in β-cells (Figures 2D,E; consequential and reflex respectively). However, they can also interact with other rhythms to create a feedback loop. For instance, the stretching rate of the blood vessel walls keeps baroreceptors updated (Figure 2B).

The three rhythms have their unique rhythmicity and they constantly interact. This is another point that we agree with the model given by Modell et al. (2015), where the signal flow is perpetual. The interactions result in rhythms that are balanced (not chaotic but not rigidly regular) observed as homeostasis in healthy biological systems.

What is interesting is that the three homeostatic rhythms may be interconnected in a broader network of trio rhythm models at different temporal scales. For example, Figures 2B,C are linked in a way that the smooth muscle cells (central rhythms in Figure 2C) are located within blood vessels (reflex/consequential rhythms in Figure 2B) as a sub-rhythmic component and Figures 2D,E are linked in a way that the clock genes (central rhythms in Figure 2E) are located within β-cells (central rhythms in Figure 2D).

In the following section, we look into selected examples of vital rhythmic phenomena in human organ systems. These examples show that taking rhythms and their interplay into consideration provides a holistic perspective of the biological rhythmic system. All three rhythms in the trio must be continuously and simultaneously active to maintain balance, and each set might be coupled to another within and across different spatial and temporal scales. Thus, the pattern may exist regardless of the size of the system, such as the human body, organ systems, or cells.

Cardiovascular rhythms

Heart rate is one of the rhythmic biological phenomena in the human body that are noticeable and crucial for sustaining life (Thaulow and Erikssen, 1991). In the regulation process of heart rates and blood pressure, baroreceptors are one of the components of the cardiovascular system that play an important role. Baroreceptors are sensors that detect mechanical properties of blood vessels that can be divided into two types: high-pressure arterial and low-pressure volume receptors (Armstrong et al., 2021). Both subtypes are stimulated by the stretching of blood vessel walls and transmit nerve impulses rhythmically to the ANS (Suarez-Roca et al., 2019; Armstrong et al., 2021). As a result of the rhythmic systole and diastole of the heart, blood vessels rhythmically and passively stretch to accommodate the pulsatile blood flow (Camasão and Mantovani, 2021). For example, when the stretching rate of the blood vessels is increased, the impulse firing rate of the baroreceptors will also be higher. Consequently, stimulation of the nucleus tractus solitarius region in the brain stem will lead to increased inhibition of cardiac output (i.e., decreased blood volume and pressure). Thus, a negative feedback loop is created that lowers the stretching rate of the blood vessel walls (Armstrong et al., 2021). This perpetual rhythmic process is called the baroreceptor reflex (Armstrong et al., 2021). Blood pressure is maintained and regulated (homeostasis; Figure 2) through interaction between the ANS (central rhythm), baroreceptors (external rhythm), and blood vessels (reflex rhythm). This interaction is one of the main components that make up the rhythmicity of the cardiovascular system. The rhythmic balance in this particular system is vital. Baroreceptors may influence blood pressure variability, and their decreased function is related to severe medical conditions such as hypertension (Tohyama et al., 2020; Ziegler, 2021).

In this model, mechanical changes of blood vessels (reflex rhythm) are due to continuous dynamic changes in blood flow depending on the blood pressure and volume that generates distension pressure on the vessel walls (Anwar et al., 2012). Apart from the baroreceptor reflex, evidence suggests that the communication between single cells is also important for synchronous rhythmic contractility of the blood vessels.

Blood vessels are a multilayered structure consisting of inner (tunica intima), middle (tunica media), and outer (tunica adventitia) cell layers (Tucker et al., 2021). Smooth muscle cells are found in the middle layer and contribute to the strength and contractility of blood vessels (Anwar et al., 2012). Interestingly, blood vessels display rhythmic activities when nerve signaling has been blocked (denervation; Siegel et al., 1991). It has been shown that this autorhythmic behavior of smooth muscle cells is regulated by intercellular communication (Koepchen, 1991; Siegel et al., 1991). This is achieved through gap junctions which are channels that permit the transfer of ions and small molecules between cells (Ross and Pawlina, 2003). Through the gap junctions, the levels of Ca^{2+} are synchronized between cells, thus resulting in autorhythmic activities (Slovut, 2004). When the gap junctions are chemically inhibited, the rhythmic activities decrease substantially (Slovut, 2004).

Taken together, a rhythmic phenomenon arises from the interaction between smooth muscle cells (central rhythm), intercellular communication (internal rhythm), and synchronization (consequential rhythm) that results in a continuation of rhythmic activities and possibly contributes to the entire cardiovascular rhythms (homeostasis; Figure 2C).

Pancreatic rhythms

The pancreas is part of the digestive organ system in the human body formed around weeks 4 and 5 of gestation (Pandol, 2010), and it consists of glands that can be largely divided into two components: exocrine and endocrine glands (Netter, 2011). Although the pancreas has been studied for many years—possibly since ancient times (Ceranowicz et al., 2015)—rhythmic activities, such as more ribosome synthesis during the day in the exocrine part of the organ, were observed and reported only a few decades ago (Volkl and Poort, 1983). Subsequently, more evidence has been accumulating to support that the pancreas is a rhythmic system. In particular, there has been growing interest in understanding more about possible correlations between circadian rhythms and core activities in the endocrine of the pancreas (e.g., insulin production and secretion), which are related to diseases such as diabetes (Marcheva et al., 2010; Sadacca et al., 2011; Vieira et al., 2013, 2014; García-Costela et al., 2020; Seshadri and Doucette, 2021).

In the pancreas, the exocrine glands help to break down nutrients by producing pancreatic enzymes, whereas the endocrine glands produce hormones, which enter directly into the bloodstream, including glucagon and insulin, to regulate the blood sugar level (Edlund, 2002). In endocrine glands, specialized groups of cells are found. These clusters, also known as the islets of Langerhans, mainly consist of four different cell types: α-, β-, δ-, and pancreatic polypeptide (PP) cells (Zhong and Jiang, 2019). Among these cells, β-cells take up the most mass of an islet (60–80%; Edlund, 2002), and they are responsible for controlling blood glucose levels by secreting hormones (i.e., insulin) in the bloodstream. Although the β-cells start to form in the early gestation stages, glucose-stimulated insulin secretion is insufficient in β-cells in neonates (Seshadri and Doucette, 2021). β-cells continue to develop during the perinatal period and show rhythmic activities only after birth.

Among various intra- and extracellular factors that are involved in the rhythmicity of β-cells (Heart and Smith, 2007; Perelis et al., 2016), rhythmic stimulation and entrainment to fasting-feeding cycles and the activation of specific circadian clock genes (*ARNTL*, *PER*, and *CRY*) may be critical factors for the postnatal maturation of β-cells (Alvarez-Dominguez et al., 2020; Seshadri and Doucette, 2021). It has been shown that the deletion of *ARNTL* (also known as *BMAL1*) inhibited the complete maturation of β-cells in rodent models (Rakshit et al., 2018). Moreover, inhibiting circadian clock genes reduced glucose-stimulated insulin secretion in β-cells even in fully matured isolated islets from both rodent and human models (Perelis et al., 2016; Saini et al., 2016). Therefore, internal and external rhythmic stimulation and entrainment of β-cells are essential for insulin secretion both in immature (during the perinatal period) and mature cells.

As an external rhythmic stimulation, the circadian rhythmic variation in melatonin protein level plays a crucial role in regulating insulin sensitivity and secretion by β-cells. Melatonin is also referred to as *Zeitgeber*, the German word for "time giver" (Pandi-Perumal et al., 2006). It is produced and secreted predominantly from a small endocrine gland in the brain called the pineal gland (Pandi-Perumal et al., 2006). However, other parts of the human body, such as the retina and skin, can also produce melatonin (Srinivasan et al., 2009). As accumulating evidence shows that circadian rhythm is an important factor in type 2 diabetes, although further investigation is necessary, it has been hypothesized that melatonin may have a therapeutic property in treating type 2 diabetes (Sharma et al., 2015). This is in line with the results from separate experimental studies. For example, treating isolated islets from rodents with melatonin overnight to mimic an *in vivo* environment promoted subsequent insulin sensitivity and secretion the following morning (Kemp et al., 2002). In a separate experimental study, a similar relationship between insulin secretion and melatonin in human type 2 diabetic patients was found: patients with decreased insulin secretion and glucose tolerance had reduced melatonin productions (Pourhanifeh et al., 2020). In this specific context, homeostatic interaction arises between β-cells (central rhythm), melatonin (external rhythm), and insulin sensitivity and secretion (reflex rhythm), which is one of the mechanisms that leads to the constant maintenance of glucose level (homeostasis) in the human body (Figure 2D).

Aside from extracellular rhythms involved in insulin secretion, such as melatonin rhythms, isolated β-cells show cell-autonomous rhythms by maintaining and regulating the rhythmic insulin secretion independently (Pulimeno et al., 2013; Perelis et al., 2015). This endogenous rhythmicity is known as basal insulin secretion (Saini et al., 2016). Basal insulin secretion is only between 0.5 and 1.0 units per hour and seems insignificant compared to the total amount of about 40 units of insulin secreted in an adult in a day (Ramchandani et al., 2010). However, basal secretion occurs continuously during the fasting periods throughout the day and accounts for approximately 50% of the total daily insulin secretion (Ramchandani et al., 2010). In *ex vivo* and *in vitro* models of the pancreatic islets, master circadian clock genes called *CLOCK* and *ARNTL* regulate synchronous cell-autonomous rhythms (Perelis et al., 2015; Saini et al., 2016). These clock genes regulate intracellular Ca^{2+} signaling pathways; the disruption of normal functions of *ARNTL* results in the inhibition of intracellular Ca^{2+} rhythms (Cavieres-Lepe and Ewer, 2021). The Ca^{2+} rhythm is an essential factor in insulin secretion and at the level of individual β-cells, receptor-mediated glucose uptake generates increased levels of adenosine triphosphate (ATP), leading to membrane depolarization followed by the opening of Ca^{2+} channels. Consequently, this influx of extracellular Ca^{2+} into β-cells activates the insulin secretory machinery and release of insulin from the cells (Campbell and Newgard, 2021). Synchronized rhythmic insulin secretion from a group of β-cells within an islet is mediated by Ca^{2+} flux through the gap junction channels that connect adjacent β-cells (Daraio et al., 2017; Idevall-Hagren and Tengholm, 2020). Accordingly, there is a possible interaction between the clock genes (central rhythm), Ca^{2+}

signaling (internal rhythm), and synchronous insulin secretion (consequential rhythm) that contributes to the continuous regulation of glucose levels (homeostasis; Figure 2E).

Biological rhythms in the context of mechanical stimulation of cell cultures

Given the above, biological rhythms are regulated both endogenously and exogenously, which occur cooperatively to regulate complex biological processes and maintain homeostasis in the system. We have classified these rhythms as a trio involving central rhythms, internal/external rhythms, and reflex/consequential rhythms, and the connections between the rhythms are essential. We will now discuss the relevance of biological rhythms in the context of mechanical stimulation of cell cultures.

When mechanically stimulating cell cultures for tissue engineering and regenerative medicine, it is necessary to consider mechanical parameters, which take place in the position of external rhythms in Figure 2A, as rhythmic variables and not as "static" variables. Rhythmic mechanical stimulation of cells could be organized as micro-rhythms (milliseconds, seconds, and minutes; ultradian rhythms) and macro-rhythms (~24 h and days; circadian and infradian rhythms). Here, we discuss the importance of this consideration in relation to the fact that the cellular mechanical response and sensitivity, which take place in the position of reflex/consequential rhythms in Figure 2A, are rhythmic (Thompson et al., 2020), rapid (ion channel activation; Matthews et al., 2010), and reduced over time (aging; Yang et al., 2017).

Firstly, cellular mechanical sensitivity and response can be rhythmic. It has been shown previously that cellular clock genes can regulate mechanical cellular functions such as cell migration—directed cell movement or change of position—in fibroblasts (Hoyle et al., 2017), resulting in showing patterns in cell migration over time. One of the cellular mechanisms that is actively involved in cell migration and mechanosensing is the cytoskeleton—a cellular component that is mainly responsible for the mechanical and structural aspects of the cells (Dominguez and Holmes, 2011). The dynamic structural alterations of F-actin filaments—a subcomponent of the cytoskeleton—in the form of lamellipodia and filopodia drive the migration at the cell front (Krause and Gautreau, 2014). Interestingly, the dynamics of F-actin filament formation can also be rhythmic. This is evident through the rhythmic intracellular expression of cofilin (Hoyle et al., 2017), a protein that regulates actin dynamics (Bravo-Cordero et al., 2013). Another example of rhythmic activities of the cytoskeleton is the fluctuating rate of wound healing which exhibits circadian rhythms where wounds (fibroblast cell cultures, skin wounds in rodents, and burn injuries in humans) are healed faster during the daytime (Hoyle et al., 2017). Furthermore, Ihara et al. (2017) illustrated that clock genes, such as *CLOCK* and *ARNTL*, can regulate the mechanosensing of the mucosa in the bladder of rodents. Healthy rodent models showed rhythmic expression of the mechanosensors, *Connexin26* (*Cx26*) and vesicular nucleotide transporter (*Vnut*), in the mucosa, which is more active during the day than at night. The disruption of the clock genes resulted in disturbed rhythmicity of the mechanosensing of *Cx26* and *Vnut* and showed an abnormally sensitive bladder during the night (Ihara et al., 2017).

Secondly, cellular sensitivity and response to mechanical stimulation can be rapid. In the process of fast cellular mechanosensing, integrins—transmembrane proteins that mediate the adhesion of cells to the extracellular matrix—play a central role (Chen et al., 2017; Martino et al., 2018). Integrins are also essential components of the focal adhesion (FA) points—multiprotein complexes that link the extracellular matrix to the actin filaments of the cytoskeleton (Wu, 2007). Generally, mechanotransduction—intracellular conversion of sensed mechanical stimulus into electrochemical signals—of integrins occurs within 500 ms after the cells were mechanically stimulated (Strohmeyer et al., 2017). However, it has been shown that the initiation of integrin-mediated intracellular Ca^{2+} influx happens as prompt as four milliseconds after mechanical stimulation was applied directly to the integrins, although the Ca^{2+} influx only peaked around 300 to 400 ms after the mechanical stimulation (Matthews et al., 2010). Moreover, integrin-mediated activation of the FA protein *SRC*—a signaling protein involved in cellular processes like migration, division, and differentiation—has been shown to take around 300 ms (Na et al., 2008). These dynamic and fast-responding cellular mechanisms are closely interrelated with cellular rhythms. For instance, *NR1D1*—a circadian rhythm clock gene—regulates FA formations in fibroblast cultures (Cunningham et al., 2020). Additionally, changes in the mechanical stiffness of the microenvironment are sensed by FA complexes and can lead to both altered circadian rhythmicity in mammary and lung epithelial cell cultures (Yang et al., 2017) and changes in rhythmic Ca^{2+} signaling between smooth muscle cells (Stasiak et al., 2020).

Thirdly, rhythmicity in cells and tissues dampens with age, which has been suggested to be partially due to the stiffening of tissue (Yang et al., 2017). The stiffening of *in vivo* tissue has a significant impact on the homeostasis of the human body in general (Sherratt, 2013; Heinz, 2021; Ryu et al., 2021). By using *in vitro* models, the substrate or extracellular matrix stiffness can be altered to mimic the physiological changes observed during aging to illustrate reduced rhythmicity. Accordingly, mammary and lung epithelial cells grown in a soft microenvironment (3D culture with stiffness ~30 Pa) exhibited functional circadian rhythmicity, whereas cell cultures grown in a hard microenvironment (2D culture with stiffness >100 MPa) exhibited reduced rhythmicity (Yang et al., 2017).

Collectively, there is adequate evidence to show that cellular processes of mechanotransduction are rhythmic, and these rhythmic cellular processes may occur in different temporal scales (micro- and macro-rhythms). A deeper understanding of mechanotransduction is crucial since many diseases arise from cellular mechanotransduction defection (Jaalouk and Lammerding, 2009). The effects of micro- and macro-rhythmic

mechanical stimulations have been reported in recent experimental findings. For instance, Rogers et al. reported on the effect of rhythmic mechanical stimulation using a flexible silicone growth substrate stretching at regular rhythmic intervals (the frequency of 1 Hz) on human stem cell cultures. Following cycles of 12 h of regular rhythmic stretching and 12 h resting period for three days, they demonstrated synchronization of the clock genes (*ARNTL*, *PER1*, *PER2*, and *NR1D1*) in human stem cells (Rogers et al., 2017). Moreover, Vágó et al. demonstrated the effect of rhythmic mechanical stimulation using a uniaxial compression force on chondroprogenitor cells (from chicken). The stimulation was one h/day for six days and rhythmic mechanical stimulation entrained circadian clock genes (*ARNTL*, *CRY1*, and *PER3*), leading to enhanced tissue homeostasis and histogenesis (Vágó et al., 2021). In both studies, the trio rhythm model has been demonstrated: synchronization of specific clock genes (central rhythm), rhythmic mechanical stimulation (external rhythm), and cellular responses (e.g., stem cell differentiation capability and tissue homeostasis; consequential rhythms).

The regulation of clock genes without chemical or temperature-related stimuli potentially increases the utility of tissue engineering research in terms of cell transplantation, apart from personalized medicine and drug screening, even further. In particular, a clock gene such as *ARNTL* is reported to be an important factor in the WNT signaling pathway (Guo et al., 2012), which is a crucial stem cell mechanism that initiates the differentiation process—a stem cell process when its potential is lost and forms into adult cells, for example, cardiac muscle cells or skin cells. These findings are encouraging, but it must be noted that stem cells from different locations in the body respond differently to the same type of rhythmic mechanical stimulation (Rogers et al., 2017). Therefore, more extensive studies on optimization of the rhythmic mechanical stimuli that can closely mimic *in vivo* cellular environments for cell cultures are necessary.

In particular, microphysiological systems or organ-on-chips provide a great advantage in growing and studying cellular responses in dynamic cell culture systems (Wikswo, 2014). Compared to conventional static cell culture systems, implementing cell cultures in microfluidic chip systems offers the possibility to mimic key aspects of human physiology more accurately, including rhythmic stimulation (Zhang et al., 2018). Thus, growing cells in micro-devices allow to control (magnitude and rhythmicity), mechanical forces (e.g., stretching, pulling, compression, and shear forces), chemical signaling (e.g., growth factors, hormones, and nutrients), and electrical stimulation that the cells are exposed to (Ergir et al., 2018).

Conclusion

In this article, we have classified biological rhythms using a trio model (central rhythms, internal/external rhythms, and reflex/consequential rhythms). It is imperative that all three rhythms in the trio function continuously to regulate homeostasis in a given biological system. Thus, the link between the three rhythms is important and relevant in tissue engineering and regenerative medicine as rhythmic interactions, whether in micro- or macro-rhythms, are vital in the early development of endogenous biological rhythms. This is evident through the lack of endogenous rhythms (e.g., transcriptional-translational feedback loop) in embryonic stem cells in general compared to adult stem cells (e.g., bone marrow mesenchymal stem cells; Rogers et al., 2017). For instance, circadian rhythmic patterns observed in neonates' heart rates disappear shortly after birth and return only 3 to 4 weeks later (Ardura et al., 1997). The rhythmicity observed in the early neonatal stage is presumed to be due to maternal influence and endogenous rhythmicity is fully developed only at a later stage, but there are conflicting views on whether external rhythms (e.g., light and dark cycles) have any effect on the development of circadian rhythms in neonates (Begum et al., 2006). Moreover, it has been shown previously that insulin secretion by pancreatic β-cells depends on external rhythms (e.g., fasting and feeding cycles) to develop endogenous rhythms and to be fully matured (Seshadri and Doucette, 2021). As these experimental observations illustrate, the importance of interactions between the rhythms in the entrainment and development of fully functioning biological rhythms should not be minimized.

As the name suggests, the trio rhythms are like a musical ensemble in that each rhythm is individually important, but they attentively listen and interact with one another and even with the audience to achieve a successful performance. Furthermore, as the ensemble starts to interact with other ensembles, it becomes a structure like an orchestral ensemble where the interactions between different sections of the orchestra are extremely sensitive and intricate to form some kind of homeostasis usually led by a conductor. The model is intentionally reductive and can be made more specific by exploring additional physical examples such as the seven-step model (stimulus, receptor, input, integrating center, output, effector, and response) presented by Chirillo et al. (2021). Still, we think it is also essential to try to pin down the core patterns from complex processes to get an overview and understanding of the discussed rhythms in this article at a macroscale. We hope that the trio model provides a framework that makes it possible to focus in and out of different spatial and temporal scales to get a basic but fundamental understanding of how biological, particularly cellular, rhythms function and interact with one another.

Author contributions

DK, AD, and AJ contributed to the conception of the study. DK wrote the first draft of the manuscript. All authors contributed to the article and approved the submitted version.

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Conflict of interest

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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Paper III

A mini acoustic chamber for small-scale sound experiments

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III

A mini acoustic chamber for small-scale sound experiments

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ABSTRACT

This paper describes the design and construction of a mini acoustic chamber using low-cost materials. The primary purpose is to provide an acoustically treated environment for small-scale sound measurements and experiments using ≤ 10-inch speakers. Testing with different types of speakers showed frequency responses of < 10 dB peak-to-peak (except the "boxiness" range below 900 Hz), and the acoustic insulation (soundproofing) of the chamber is highly efficient (approximately 20 dB SPL in reduction). Therefore, it provides a significant advantage in conducting experiments requiring a small room with consistent frequency response and preventing unwanted noise and hearing damage. Additionally, using a cost-effective and compact acoustic chamber gives flexibility when characterizing a small-scale setup and sound stimuli used in experiments.

CCS CONCEPTS

• **General and reference** → Experimentation.

KEYWORDS

Mini acoustic chamber, sound insulation, sound measurement, acoustic experiment

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Figure 1: Fully constructed mini acoustic chamber. (Top left) Side view of the bottom part of the top cover. (Top right) Top view of the top cover. (Bottom left) Inside of the chamber. (Bottom right) Fully assembled chamber. The two handles are used to lift open the top cover. The round hole in the center for accessing the chamber for measurement (e.g., microphone) and observation (e.g., digital microscope)

1 INTRODUCTION

Research involving sound measurements often require a room with acoustic treatment, such as an anechoic chamber. However, professional anechoic chambers are not readily available and are costly to build. A full anechoic chamber is especially uneconomical for small-scale experiments. This is the case in our research on the effect of musical sound on biological cell cultures [12]. Our research builds on efforts to understand the mechanotransduction of cell cultures—cells grown in a controlled artificial environment in biology laboratories—induced by sound pressure waves [4]. Mechanotransduction is an essential process of the cells where mechanical signals (stimuli) are transformed into biochemical signals. In nature, cells are exposed to different types of mechanical forces such as tension, compression, and shear forces [5]. Although these mechanisms are not fully understood yet, research has shown potentially beneficial effects of audible range sound, even music in some cases, on the biological processes of the cells [12].

In a recent review study [12], we documented a high variation in the methodologies employed in investigating the effects of audible sound on cell cultures. Biological cells have a complex system that changes their behaviors by responding to chemical and mechanical cues. This requires a more sensitive approach when performing experiments using mechanical stimuli [7]. Thus it is critical to control and document the experimental setup carefully, not least to allow for replication of findings. Then it is necessary to have an acoustically controlled space for measurement and characterization

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of the sound used as stimuli for experiments [8]. Additionally, many experimental studies have employed fairly loud sound (around 90 dB Sound Pressure Level (SPL)) over several hours in some cases [4]. Exposure to such high-intensity sound can result in temporary and even permanent hearing damage to the researchers. Sound dampening devices can reduce the risk of such irreversible health hazards and protect the hearing of the researchers and staff working in the lab.

Taken together, we found the need to develop our own movable and acoustically treated "room" (Figure 1). This paper presents the design and construction of a custom-built mini acoustic chamber. We call it "acoustic," since the aim has not been to make an anechoic chamber. An anechoic chamber is designed to mimic an infinitely large room where there is no reflection of sound, minimal or no external noise entering the chamber, and where the sound generated inside does not escape the chamber [8]. Several studies have aimed at developing small [1, 2, 9, 13–15] and economic anechoic chambers [8, 10]. More recently, Jameeu et al. developed a mini anechoic chamber for antenna (electromagnetic) measurement [11]. We have been inspired by these approaches when constructing our mini acoustic chamber using materials that can be purchased at local stores.

Our intention is to pave the way for a controlled experiment environment that provides a relatively "flat" and consistent frequency response and controlled noise and loudness level. We hope this can promote controlled small-scale acoustic measurements and experiments. Our target application is sound studies on cell cultures, but the chamber could also be used for other applications. This paper illustrates the design, construction, and characterization of such a mini acoustic chamber.

2 CHAMBER DESIGN

2.1 General consideration

The shape of the chamber was one of the critical factors when we embarked on designing the chamber. After searching through relevant literature, we found that a rectangular-shaped chamber is the most practical to work with [16], although the chamber will be prone to standing waves in a higher frequency range due to its small size. Compared to more complex shapes, a rectangular shape is simpler to design, construct, and easier to use. Several other factors were considered, including chamber dimensions and net volume concerning the size and volume of test objects, absorption material types, and room modes. In the following sections, we will discuss chamber dimensions, volume, and materials used to construct the chamber.

2.2 Dimensions

The dimensions used to construct the chamber are based on the size of the test objects with respect to the ISO 3745 [6], which in our case is a standard 60 mm cell culture dish. The dimensions used for the chamber are listed in Table 1. The values were calculated according to the ISO 3745 standard [6] to provide enough horizontal space for working with the test object.

Table 1: The dimensions of the chamber

Side	Outer (cm)	Ratio	Inner (cm)	Ratio
Length (l)	33.10	1.27	23.40	1.42
Width (w)	28.80	1.10	19.10	1.16
Height (h)	26.10	1.00	16.40	1.00

Table 2: The thickness and costs (in Euro) of the materials

Material	Thickness (cm)	Cost (€/m^2)
Plywood (red temp)	2.20	33.50
Rockwool	2.00	5.80
Felt	0.50	8.20
Acoustic foam	5.00	60.50
Miscellaneous	-	60.00
Total	9.70	168.00

2.3 Volume

ISO 3745 suggests that, for a full anechoic chamber, the volume (V) of the test subject for measurement should be 5% of the net volume of the chamber: $V_{specimen} \leq 0.05 \cdot V_{chamber}$ [6]. The gross volume of our chamber is: $V_{gross} = 33.1 \cdot 28.8 \cdot 26.1 = 24,880.6\ cm^3$ and the total thickness of one side of the wall materials is 9.7 cm. The net volume of the chamber is then: $V_{net} = 23.4 \cdot 19.1 \cdot 16.4 = 7,330\ cm^3$.

A typical volume for a 60 mm cell culture dish is (radius=3 cm, height=1.6 cm): $V_{60mm} = \pi r^2 \cdot h = 45.2\ cm^3$. Therefore, the volume requirement of the ISO 3745 is then satisfied since: $V_{60mm} < 0.05 \cdot V_{net}$.

2.4 Materials

The aim was to construct a cost-effective chamber using readily available materials (Table 2). We used a computerized numerical control (CNC) milling machine[1] to cut the material to size in a public maker space. The fully constructed chamber and its parts can be seen in Figure 1.

3 CHARACTERIZATION OF THE CHAMBER

For the characterization of the chamber, we used a setup (Figure 2) with an omnidirectional calibration microphone having a flat frequency response (Earthworks M50) connected to a sound card (Behringer UMC404) and free software (Room EQ Wizard (REW) version 5.19) on a laptop. The location and types of speakers were varied for comparison.

3.1 Sweep used for the measurement

We used REW on a laptop to generate the logarithmic sine sweep from 0 to 22 kHz (from DC to 10 Hz was a linear sweep). The total time of the sweep, including silences at the beginning and end of the sweep, was approximately 6 seconds. This was based on having 256k samples in a sweep.

[1] ShopBot CNC milling machine: https://wiki.bitraf.no/wiki/Fresing

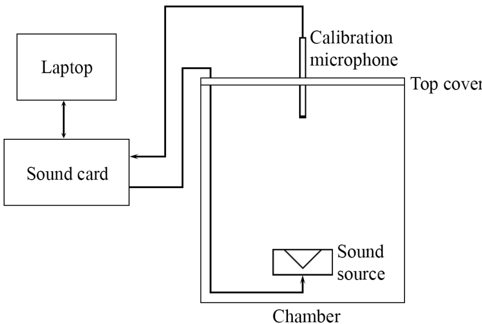

Figure 2: Flowchart of the measurement setup.

3.2 Measurement

We used three different types of speakers (sound sources) for comparison:

- Sound source 1: A surface transducer (ST) coupled with a 60mm cell culture dish
- Sound source 2: A two-way 10-inch coaxial speaker
- Sound source 3: An active two-way studio speaker (Genelec 8020b), used for sound insulation test placed outside the chamber

First, we characterized sound sources 1 and 2 using the types of speakers of suitable size for the chamber. Then we characterized the chamber using the sine sweep. We tested sound insulation efficiency using broadband noise for about 20 seconds through sound sources 2 and 3, which were kept outside the chamber. The measurement was divided into two sections and taken in the following order:

- Measurement 1: Sine sweep in the chamber with or without the chamber cover
- Measurement 2: Broadband noise with a microphone in or outside of the chamber

3.3 The modes of the chamber

A distribution of modes is crucial to avoid any heavy concentration of energy. As mentioned above, an ideal chamber would have an irregular shape but would be difficult to build. The Bolt-area indicates an accumulation of good room ratios [3] and has been tested using the inner dimensions and the ratio of the chamber on Amcoustics.com. The chamber is within the so-called "safe zone," and the modes are likely to be distributed more evenly than if the proportion of the chamber fell outside of the "safe zone."

In a rectangular-shaped space, a room mode is defined by:

$$f = \frac{v}{2}\sqrt{\left(\frac{l}{x}\right)^2 + \left(\frac{m}{y}\right)^2 + \left(\frac{n}{z}\right)^2} \qquad (1)$$

where l, m, n are positive integers that cannot be all 0. As a reference, the first five modes of the chamber can be seen in Table 3.

Table 3: The first five modes of the chamber.

Mode number	Frequency (Hz)	*l-m-n*
1	732.91	1-0-0
2	897.91	0-1-0
3	1045.73	0-0-1
4	1159.05	1-1-0
5	1276.99	1-0-1

4 RESULTS

Figure 3 summarizes the frequency responses from the experiment. In measurement 1, we observed the distinctive frequency responses of the two systems. For sound source 1, the high positive peak around 8 kHz (λ ~43 mm) is speculated to be a resonant frequency created from the coupling between the transducer (Ø = 30 mm) and the coupled dish (Ø = 60 mm). For sound source 2, the negative peak below the 2 kHz area fits the specification description from the manufacturer of the speaker.[2] The difference between the measurements with or without the chamber cover is the boosts in the frequencies below about 100 Hz when the cover is closed, effectively making our chamber "behave" like a pressure chamber.

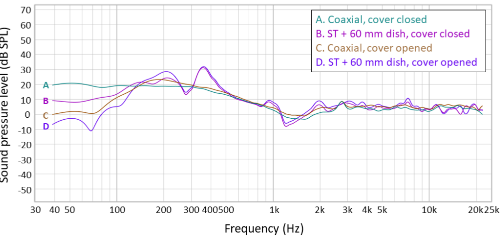

Figure 3: Acoustic chamber frequency response. The response is relatively flat above 1 kHz.

For the sound insulation effectiveness of the chamber, we compared the measured dB SPL level (measurement 2) using the room noise level as our reference. There was about a 20 dB SPL reduction when the broadband noise (sound source placed outside the chamber) was measured from inside of the chamber than when it was measured from outside of the chamber (Figure 4). We suspect the reduction could have been larger since the measurement outside the chamber was very similar to the room noise level.

An interesting and perhaps predictable finding is the frequency boosts in the range between 100 and 900 Hz in the frequency response of the chamber, as can be seen in Figure 3. The chamber's "boxiness" seems inevitable in such a confined space. The chamber frequency response's higher range (from 1 to 20 kHz) is generally constant (±4 dB SPL), and the variation is not as extreme as the "boxiness" frequency range.

[2] More information can be found on the project web page: www.uio.no/ritmo/english/research/interaction-robotics/sound-box/index.html

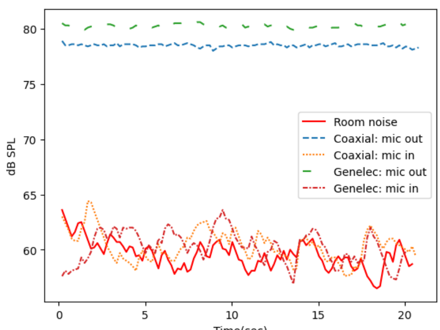

Figure 4: dB SPL measurement of broadband noise comparison. The speakers were kept outside the chamber and measured with the microphone outside and inside the chamber.

5 DISCUSSION AND CONCLUSION

The main aim of this paper was to produce an acoustically controlled space for small-scale acoustic measurements and experiments. We have described the design of a mini acoustic chamber that can be constructed at a minimal cost using a simple design for research and small-scale sound measurement or experiment purposes.

The chamber was characterized in terms of its frequency response and sound insulation efficiency using various sound sources. After the characterization, it became clear that the "boxiness" of such a small chamber is inevitable, and it should be taken into account when performing experiments. Despite the downfall, we succeeded in reaching our goals. Firstly, the frequency response of the chamber showed a fluctuation of less than 10 dB SPL and stayed approximately flat from 1 kHz up to about 20 kHz. Secondly, the sound insulation test of the chamber showed a significant reduction (approximately 20 dB SPL).

These are positive results for our purpose (acoustic experiments on biological cell cultures) and possibly for other small-scale experiments and measurements, for example, small antenna measurements. We hope that such a simple and small acoustic space, like our mini acoustic chamber, will provide a more controlled and accessible space for small-scale experiments.

The aim now is to use the chamber in experimental studies of cell cultures in laboratories to reveal any shortcomings of the constructed chamber. Knowledge from the practical work and adjustments to the ratios and dimensions of the chamber could be used to construct another chamber that is more safely within the Bolt-area. It would also be interesting to characterize the chamber without the absorption materials, which could give a better understanding of how the materials are performing.

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Paper IV

The effect of rhythmic vertical vibration of cell culture on the F-actin filament structure

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Will be submitted soon.

IV

Appendices

Appendix A

Supplementary material

A.1 Audio signal generation and manipulation

A standalone Max/MSP patch can be downloaded at https://doi.org/10.5281/zenodo.7747414.

A.2 Image processing and feature extraction algorithm

The Actin Analyzer can be downloaded at https://github.com/donghodk/actin-analyzer.

A.3 Biological data

The raw fluorescence microscopic images and feature extraction (numeric) data can be found at https://doi.org/10.18710/ALOBQK.