Chapter

Perspective Chapter: *In vitro* Contracting Cardiomyogenic Models from Whole Fish Embryos and Larvae – Method, Properties, and Applications

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Abstract

Heart diseases remain a leading cause of mortality worldwide. The development of effective treatments and interventions relies on a deep understanding of cardiac biology. Traditional two-dimensional (2D) cell cultures and animal models fall short in replicating crucial physiological and pathological features of cardiac tissue. In response, 3D cardiac models have emerged, offering a more faithful replication of the native heart tissue's architecture and functionality in a controlled environment. Although technical hurdles limit the widespread adoption of *in vitro* 3D models, they hold promise for advancing cardiovascular research. This chapter provides a description of the development of 3D spontaneously contracting cardiac primary cultures derived from fish embryos and larvae, presenting an easily accessible model for diverse applications, including the investigation of viral heart infections, as well as biomedical, pharmacological, and cardiology research. In this chapter, we will highlight the importance of *in vitro* model systems for modern cardiac research. Additionally, we will provide an overview of the protocol and results concerning the creation of *in vitro* 3D heart-like cell aggregates using enzymatically digested whole fish embryos/larvae. These aggregates exhibit long-term stability and spontaneous contractions, making them promising candidates for high-throughput screening

Keywords: cardiac model, 3D culture, heart, fish virology, pharmacology

1. Introduction

Cell cultures represent a fundamental *in vitro* technology in several research fields [1–3]. Since these experimental systems have become part of everyday research, they have contributed to important discoveries, including key findings in cardiomyocyte mechanisms concerning the cardiovascular field [3]. Cardiomyocytes are the main components of heart tissue and have their origin in mesodermal stem cells [4]. A lot of work in cardiovascular research has been focused on the development of cardiomyogenic models to study various conditions, e.g., heart diseases and healing capacity [5].

In vivo models of animal species like rats and pigs are currently used as the gold standard to understand pathology mechanisms and explore novel treatments within the context of a whole organism [3, 6], as the cardiovascular apparatus is characterized by a high degree of complexity, which means that a number of cardiovascular diseases are usually multifactorial [7, 8]. However, these kinds of experimental *in vivo* models have some important limitations that make them alone insufficient in certain circumstances such as the discovery and development of new therapies [3, 6]. The main disadvantages of *in vivo* models are in fact the ethical concerns, the high costs, and the low throughput. As an alternative, *in vitro* systems are good platforms for high-throughput drug efficiency and toxicity tests and are widely used to study biological mechanisms in a faster, low-cost, and reproducible way [6]. Taken together, *in vivo* and *in vitro* systems allowed the study of complex biological mechanisms and, during recent years, new emerging vertebrate models enriched the tools of researchers engaged in cardiovascular studies for human and veterinary medicine [9].

Since the 1990s, teleost fishes, in particular, have grown in popularity because of the introduction of zebrafish (*Danio rerio*) as a commonly used animal model in several research fields due to biological similarities with humans and generally good conservation and expression of important target genes. Specifically, zebrafish is a small freshwater cyprinid that is cheap and easy to grow and produces a high amount of external transparent embryos, making it an efficient model for multi-disciplinary studies in cardiac research, covering a range of topics including, but not limited to, development, genetic biology, and regeneration [10]. In addition to zebrafish research, in general, fish cell cultures of the mainly remunerative fish species are increasingly established due to their relevance in the important economic sector of aquaculture and fisheries, and the necessity to obtain more cost-effective model systems for research in this field as well [11].

For these reasons, multiple *in vitro* cardiomyogenic models from different mammalian and teleost species were developed for basic and applied research during the last decades [3, 9].

The technology used to produce cardiomyogenic models can vary according to the cell source and the use of 2D or 3D culture conditions. Among the variety of experimental techniques, this book chapter will focus on the cultivation methods for 3D autonomously beating cardiomyocytes (defined as "SCC—Spontaneously Contracting Cell aggregate", ZebraFish Heart AggregateZFHA, and SCPC—Salmon Cardiac Primary Cultures") using whole fish larvae from the pre- and post-hatch stage as the main cell source, including derivation outcomes from different species. In addition, the following sections explore the potential applications of 3D spontaneously beating cardiomyogenic fish cell cultures, with emphasis on basic research regarding their use to study resilience to climate change, and in applied research in pharmacology and fish virology.

1.1 Overview of cardiomyogenic *in vitro* models

The main approaches employed to generate *in vitro* heart models can be categorized based on the type of biological sample utilized. These include the utilization of whole isolated heart organs [12], sections of heart tissue [13], explant cultures [14], cultivation of induced pluripotent stem cells (IPS) [15], as well as the culture of embryonic stem cells, and embryoid bodies [16]. However, if these approaches are carried out using adult heart tissue from mammals, the regenerative capacity is not sufficient. In addition, obtaining biological samples from humans is challenging due to costs and ethical considerations.

Furthermore, the general limited availability of differentiated cardiomyocytes in 3D models poses a significant constraint in cardiovascular research. A promising tool for research involves the use of induced pluripotent stem cells to generate fully developed cardiomyocytes. Nonetheless, this technology is still in its early stages and faces limitations in terms of differentiation efficiency, thereby reducing the model's reliability [17]. Over the past few years, a growing body of research has focused on utilizing cardiac scaffold-free spheroids for drug testing and toxicology purposes. Numerous studies have been published during this time, often employing a combination or co-culture of various cell types including rodent or human primary cardiomyocytes, human induced pluripotent stem cell-derived cardiomyocytes (hiPSC-CMs), fibroblasts, stem cells, and endothelial cells [17–23]. An inherent problem with previous *in vitro* cardiac test systems derived from various organisms such as mice, birds, amphibians, and fish is their limited activity. These test systems show either no contraction or only in response to electrical stimulation or only brief contractions, lasting from a few minutes to a few hours or days, which limits their use in the study of contractile activity of cardiac tissue [24]. Another disadvantage of conventional cell cultures is their lack of resemblance to organotypic structures because, in most cases, *in vitro* models are available as individual cells or as adherent cells organized in two-dimensional layers.

It is widely recognized that the physiological environment of cells is characterized by the presence of an extracellular matrix and complex interactions based on biochemical and mechanical stimuli [1, 3, 17]. Specifically, cardiomyocytes are naturally and constantly subjected to shear stress from blood flow, mechanical deformations, electrical impulses, and calcium transients, which are mostly absent in the artificial environment of classical two-dimensional (2D) cell cultures. For these reasons, there is a need to achieve experimental three-dimensional (3D) models with the ability to mimic the complex and dynamic microenvironment found in living tissues [17].

The advantages of 3D cell cultures over conventional 2D cell cultures are manifold. In 3D cultures, cells can interact with each other and their environment, allowing for the formation of cell-cell contacts, cell-matrix interactions, and the development of tissue-like structures. In particular, cells in 3D cultures can communicate more effectively through direct cell-cell contacts such as gap junctions. This enables the exchange of important molecular signals, growth factors, and cytokines, leading to improved cell differentiation, functionality, and responsiveness to external stimuli. This better mimics the conditions and cellular behavior *in vivo*, leading to more accurate and reliable experimental results.

Consequently, 3D cultures provide a valuable platform for the study of cell–cell interactions, tissue development, disease progression, and drug treatments [17, 22].

Since 2006, there has been a growing interest in utilizing fish cells for a more detailed study of cardiac physiology and human cardiac research [24–26]. Researchers have highlighted significant differences between murine and human cardiac electrophysiology, while noting the remarkable similarity between fish and human cardiac electrophysiology in terms of action potential properties and thus ion channels [24, 25]. Compared to the human models, 3D spontaneously beating cardiomyogenic cultures from fishes are not dependent on clinical samples, and several experimental analyses confirmed the presence of fully developed cardiomyocytes organized in adult tissue phenotype and owning many biological characteristics in common with human cardiomyocytes [27–34]. The establishment of *in vitro* model systems with fish cells is therefore important not only for basic research in fish biology and virology but also for research in human medicine and pharmacology.

2. Establishment and cultivation of 3D spontaneously beating heart aggregates from fish

It is possible to obtain autonomously contracting 3D cardiomyogenic aggregates from both pre- and post-hatching whole fish larvae in a simple and efficient way (**Figure 1**). The main lab equipment and materials needed are in line with common primary cell culture isolation protocols and are summarized in **Tables 1** and **2**. At this point, it is significant to use larvae that are at least at eye point stage and still within the yolk sac phase so that they are not actively feeding. Larvae of several salmonid species like rainbow trout (*Oncorhynchus mykiss*), brown trout (*Salmo trutta*),

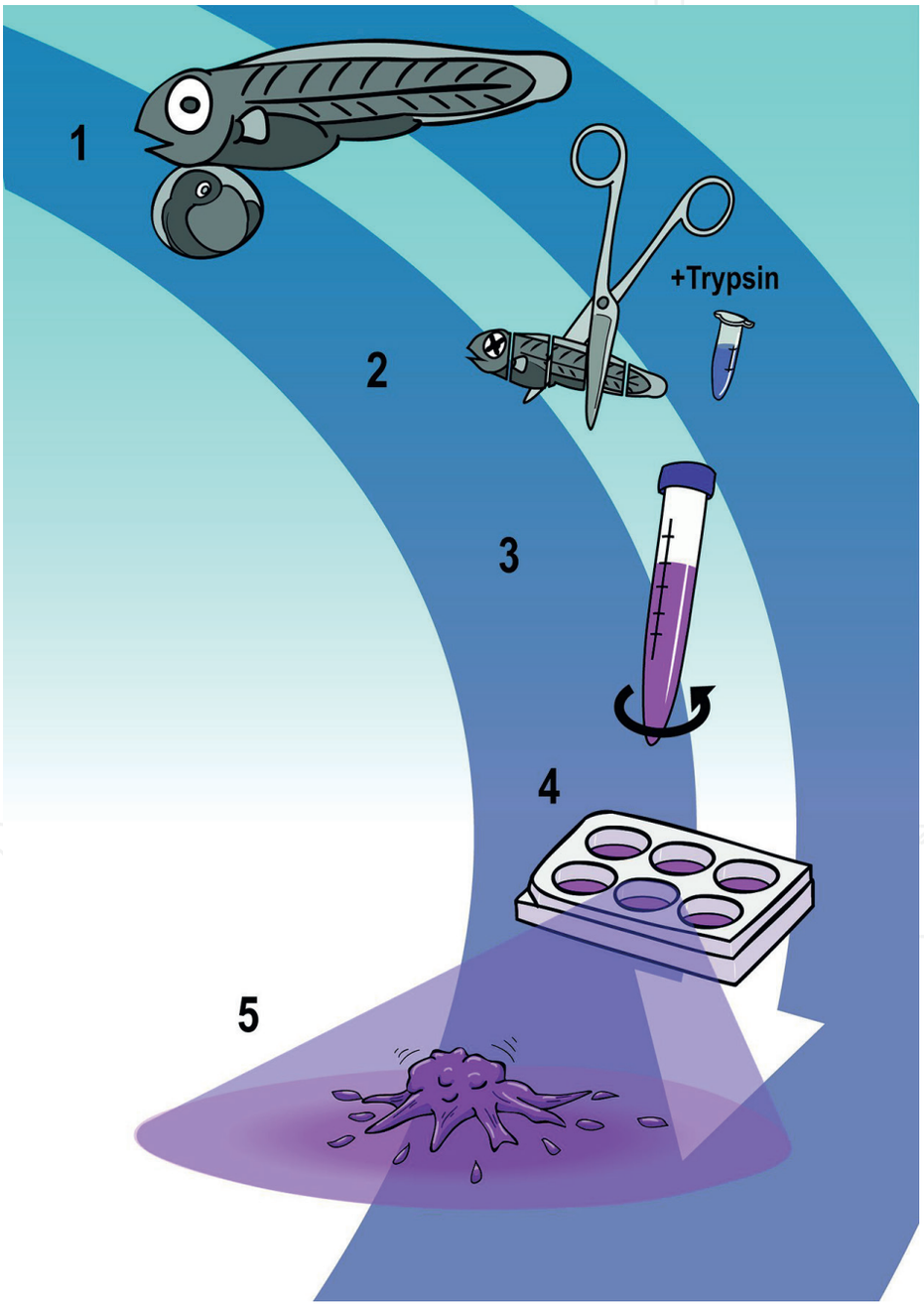

Figure 1.
Illustration of the establishment of spontaneously contracting cell aggregates (SCCs)/ZebraFish heart aggregates (ZFHAs) and Salmon cardiac primary cultures (SCPCs).

Category	Equipment
Lab equipment	Laminar Flow Sterile workbench Autoclave Sterilizator Incubator (for temperatures between 15 and 30°C) Centrifuge Microscope
Instruments	Forceps Scissors Pipettes Pipetboy
Solutions/reagents	Fetal calf serum Cell culture medium (Leibovitz 15 or DMEM) Phosphate-Buffered Saline Solution (PBS) Amphotericin Gentamycin Kanamycin Penicillin/Streptomycin Trypsin–EDTA Solution
Cell culture plastic	Pipette tips (from 10 μl up to 5 ml) Cell culture plates (6-well up to 24-well plates) Falcons (50 ml and 15 ml) 1.5 ml vials

Table 1.
Overview of the cell lab equipment required for the establishment of SCCs, ZFHA, and SCPCs.

Protocol step	Material	Name
Washing	Washing solution	Dulbecco's phosphate-buffered saline (DPBS)
Dissociation	Enzymatic solution	Trypsin solution 0,1%
		Trypsin solution 2,5%
Centrifugation	Neutralizing solution (culture medium + FBS)	Dulbecco modified eagle medium (DMEM)
		L-15 medium (Leibovitz's)
		Fetal Bovine Serum (FBS)
Cell seeding	Multi-well plate	6-well culture plate
		12-well culture plate
		24-well culture plate
	Culture medium	Dulbecco modified eagle medium (DMEM)
		L-15 medium (Leibovitz's)
	Culture supplement	Fetal Bovine Serum (FBS) 20%
	Antibiotics	Penicillin (100 U/ml)
		Streptomycin (0.1 mg/ml)

Table 2.
Summary of the main solutions/reagents used for the establishment and cultivation of SCCs, ZFHA, and SCPCs divided per protocol step.

maraena whitefish (*Coregonus maraena*), Atlantic salmon (*Salmo salar*), but also zebrafish (*Danio rerio*) or sturgeon species like *Acipenser oxyrinchus* were used to produce these cardiomyogenic *in vitro* systems [27–34].

1. Depending on the pre- or post-hatched larvae, the specimen needs to be isolated out of the egg. The eggshell is gently scored and pulled apart, ensuring the well-being of the animals. Subsequently, in accordance with ethical guidelines and animal welfare regulations, the animals are euthanized. After euthanasia, the larvae are washed three times in PBS (phosphate-buffered saline) to reduce contamination.

2. The most common enzymatic dissociation protocol involves the use of 0,1% trypsin solution for 1 to 3 minutes at room temperature, together with the induced mechanical dissociation using scissors. Depending on the fish's size, the size of the scissors needs to be adapted; for ZF larvae, micro-scissors were used.

3. The digestion is then stopped with the addition of the double or triple amount of culture medium with 20% fetal bovine serum (FBS). The immediately following centrifugation for 5 minutes at around 130 g allows the collection of isolated cells and small explants from the processed larvae. The growth medium used is alternatively Dulbecco modified eagle medium (DMEM) with 20% FBS or L-15 (Leibovitz's) medium with 20% FBS, both added with 100 U/ml penicillin and 0.1 mg/ml streptomycin.

4. Depending on the size of the fish larvae and their growth potential, primary cell cultures are then cultivated in 24/12/6-well plates. Cells from salmonids and sturgeon can be cultivated in a six-well plate, and from smaller fish species like the zebrafish, the use of a 12 or 24-well plate is preferable. Additionally, the cultivation temperatures depend on the species: cells of cold-water fish species, like Atlantic salmon, rainbow trout, maraena whitefish, or Atlantic surgeon, should be cultivated at 15–22°C, and warm-water fish species like the zebrafish at 28°C.

5. After isolation, explants and cells are generally attached after 24–48 h. Using the described protocol, the generation of spontaneously and autonomously beating cell aggregates, experimentally identified as fully developed cardiomyocytes, was possible for all the cited species (Video 1, https://bit.ly/3SRb9Wb).

2.1 Troubleshooting—contamination

Contamination in primary fish cell cultures poses a significant challenge to maintaining the integrity of research experiments and ensuring the reliability of results/outcomes. Here, we outline a systematic and scientific approach to mitigating and preventing contamination, both bacterial and fungal. Before initiating cell cultures, clean and sterilize all equipment and glassware. Just work under the sterile bench and use gloves that were previously cleaned with a 70% ethanol solution. Additionally, the contamination can be avoided/prevented or reduced through careful washing steps before preparation.

Upon identification of contamination, take the following steps:

1. Avoid cross-contamination by using separate sterile pipettes and tools for each well.

2. Wash the affected culture wells twice with sterile PBS. If fungal contamination is localized in single spot, use a sterile pipette to carefully aspirate fungal material from the contaminated culture well.

3. Initially, supplement the culture medium with double amount of the standard antibiotic concentration for the first few days.

4. If contamination persists or worsens, consider discarding individual contaminated wells while preserving uncontaminated cultures.

5. Implement a rigorous regimen of washing every 2 days and maintaining the elevated antibiotic concentration for at least 1 week.

6. After this period, gradually reduce the antibiotic concentration while closely monitoring cultures.

7. Continue monitoring for a minimum of 3 weeks to ensure complete eradication of contaminants.

2.2 Troubleshooting—absence of cardiomyogenic aggregates

The isolation protocol described in Section 2 is generally easy to perform compared to common cell isolation practices, but good technical execution is nevertheless essential to ensure the presence of functionally active cardiomyogenic aggregates. The following are some tips to consider in order to maximize the yield of heart aggregates during the isolation process.

1. Dissociation technique: When performing dissociation, ensure that you target the specific area of the original heart in small pieces. Cardiomyogenic aggregates are believed to originate from the original heart progenitor cells. Therefore, the likelihood of achieving a high yield may depend on the preservation of the portions of the original embryonic heart (refer to Chapter 3.1 for more details).

2. Overzealous microscopy examination: It is crucial to limit the frequency of microscopy inspections. Frequent disturbances by removing cultures from the incubator can decrease the occurrence of contracting aggregates. Cells require a period of rest to develop and maintain these structures. Therefore, just check maximum once per day.

3. The wells must be carefully examined at an appropriate speed to discern contractions.

3. Characteristics

3.1 General properties

A first work in 2010 proved the possibility of obtaining SCCs from rainbow trout by applying the previously described protocol [27]. In rainbow trout, it was shown that a short time before hatching, the highest ratio of SCCs can be gained with up to five SCCs from one fish larva [29]. In zebrafish, here called zebrafish heart aggregate (ZFHA), larvae should be used 3-4 days post-fertilization (dpf) [31].

The 3D SCCs are able to mimic myocardial tissue organization after a few days in culture. After 7 days in culture, most of the SCCs are developed [29]. Analysis of

SCCs, larval heart, and adult fish heart suggested that the embryonic progenitors of heart tissue are able to differentiate *in vitro* in fully developed adult-like cardiomyocytes while the heart from larval stage revealed immature and unorganized sarcomere structures within the cells, so that heart cells of larvae are not fully differentiated. These kinds of observations were verified by electron microscopic analysis for SCCs generated from salmonids [29, 32]. Additional confirmation of the theory was performed with zebrafish Tg (cmlc2:eGFP) larvae whose fluorescence indicates the expression of eGFP attached to cmcl2 in the heart [34]. This fluorescence signal was also present in the *in vitro* ZFHA indicating the further differentiation and propagation of heart progenitor cells [34].

This work, in particular, demonstrated the possibility of cultivating active cardiomyocytes from rainbow trout or other fish species in the long term over several months of constant beating frequency, **Figure 2** [29]. These important results show the possibility of obtaining highly specialized adult-like cardiac structures from whole larval cell cultures and the possibility of using fish heart aggregates as *in vitro* specification models in developmental biology.

3.2 Cell anatomy

The cell anatomy of SCC, which includes various cell types of the heart, has been extensively characterized using advanced imaging techniques such as confocal microscopy and electron microscopy, providing detailed information about the ultrastructure and spatial organization of cells [27–34].

Transmission electron microscopy confirmed the presence of well-developed sarcomeres within the beating cell aggregates with a length ranging from 1.4 to 1.6 μm consistent with adult cardiac tissue structure [27, 29, 31]. Moreover, the reported presence of T-tubuli structures in the z-bands and near the sarcoplasmic reticulum indicates the importance of ion flux and action potential [29].

Moreover, immunohistochemical analysis within SCCs revealed the presence of well-organized sarcomeres and heart-specific proteins [27–31]. LC-MS mass spectrometry analysis confirmed the previously reported observations by showing that 80% of the isolated proteins from 3D SCCs/ZFHA were also expressed in the atrium

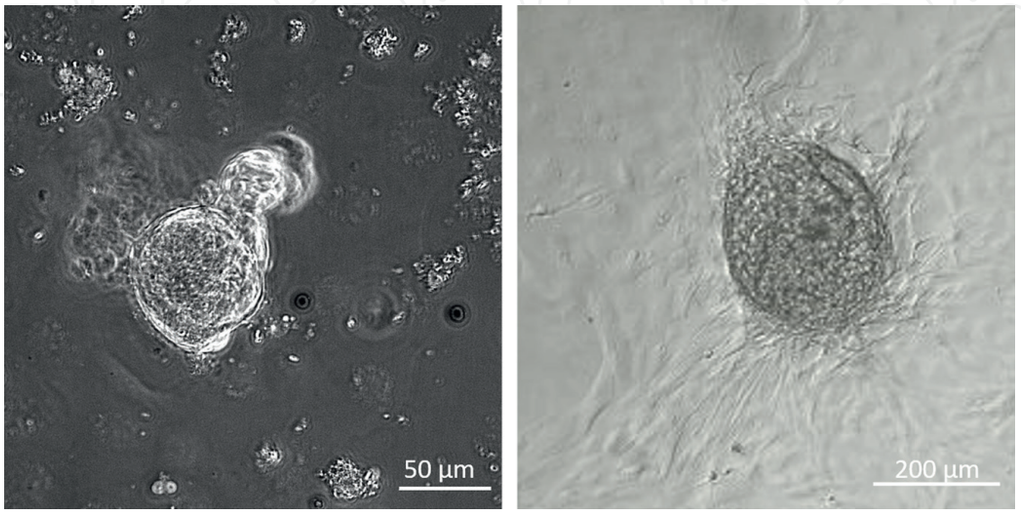

Figure 2.
SCC established from zebrafish (Danio rerio) and rainbow trout (Oncorhynchus mykiss).

and/or ventricular tissue of adult animals of the same species [31]. Studies on SCCs generated from larvae of zebrafish, rainbow trout, and Atlantic salmon have demonstrated important aspects of cell morphology within SCCs [27–34]. In fact, it has been shown that *in vitro* heart aggregates exhibit a syncytium-like structure with a high number of mitochondria, cell contact, and myofilaments, reflecting their contractile capacity, active metabolism, and long-term stability [27–34].

Pacemaker cells are the primary contractile cells responsible for generating the long-lasting rhythmic contractions observed in SCCs. Two important proteins associated with pacemaker cell function are HCN4 (Hyperpolarization-activated cyclic nucleotide-gated channel 4) and Connexin45 (Cx45). HCN4 is a key ion channel protein, which is responsible for the "funny" current (If), which plays a critical role in the generation of pacemaker potentials and the spontaneous depolarization phase of the action potential. Cx45 is a gap junction protein that facilitates electrical coupling between pacemaker cells and helps synchronize their activity. Both have been found in the SCCs [28].

Taken together, these results suggest the high reliability of SCCs in the *in vitro* reproduction of heart tissue development and organization, highlighting their potential use as 3D cardiac model systems.

3.3 Cell physiology

In vitro systems featuring functional ion channels serve as invaluable resources for scientific research. Ion channels, which are transmembrane proteins forming pores, facilitate the movement of electrically charged particles (ions) across cell membranes. They play a crucial role in regulating electrical signaling within cells. By employing *in vitro* systems that replicate ion channels, researchers gain the ability to investigate their functionality and regulation under carefully controlled conditions [35].

Electrophysiological techniques enable the recording and analysis of electrical signals generated by cells. Intracellular action potentials refer to the electrical impulses that occur within individual cells, while extracellular field potentials represent the summation of electrical activity from multiple nearby cells. These measurements provide valuable insights into cellular communication, ion channel function, and tissue-level electrical properties. Electrophysiological measurements of intracellular action potentials and extracellular field potentials thus provide valuable information about cellular electrophysiology. These techniques have multiple applications and contribute to our understanding of cardiac function in general, and more specifically arrhythmias, ion channelopathies, and drug effects on cardiac cells [36].

In the case of SCCs, several studies have demonstrated their electrophysiological properties. These studies have shown that SCCs can beat autonomously for up to 6 months without electrical stimulation. Electrophysiological analysis using sharp electrodes revealed that the cardiomyocyte characteristics of the SCC are very similar to human cardiomyocytes *in vivo* [28, 30]. The action potential exhibits a distinctive plateau phase and a rapid final repolarization phase. Initial tests have shown the reaction on several drugs (please see Section 4.2.1.) as well as on temperature. The contraction frequency of the SCCs correlated highly with temperature [29]. A steady and significant increase in their frequency with increasing temperature and vice versa was observed [29], as found in the *in vivo* condition in fish and mammals [37–39].

Overall, *in vitro* systems and electrophysiological measurements in fish models offer valuable insights into ion channel function, cardiac electrophysiology, and drug effects. These models contribute to our understanding of cardiac disorders and aid in the development of therapeutic interventions in human and veterinary medicine.

4. Applications

In line with the described properties of Section 3, SCCs represent a potential investigative tool in numerous research fields and allow the study of different aspects of cardiac tissue, which are consistent with its complexity. The following is a nonexhaustive description of some of the main potential areas of application according to the experimentally proven properties of SCCs to date.

4.1 Basic science: fish heart research

4.1.1 Climate model

In vitro cardiac models are valuable tools for studying the impact of climate change on cardiac health and function [40–43]. These models enable researchers to investigate the effects of various environmental factors associated with climate change on the heart in a controlled laboratory setting. This includes exploring the effects of temperature fluctuations and changes in oxygen levels on cardiac cells, allowing researchers to manipulate temperature and oxygen concentrations to simulate hypoxic or hyperoxic conditions to overall mimic different climate scenarios *in vitro*. In this way, the effects of temperature and oxygen changes on cardiomyocyte viability, contractility, electrical activity, and overall cardiac function can be assessed, allowing further investigation of the biological mechanisms underlying cardiomyocyte behavior under adverse environmental conditions [37–44]. Moreover, extensive research has demonstrated the influence of temperature on cardiac contractility across vertebrates [24, 37, 41, 42]. Therefore, the need to use *in vitro* heart model in the field of climate change is also of great importance to researchers working on fish health, aquaculture, and biodiversity conservation. Indeed, ectothermic animals experience temperature changes due to their environment that result in a significant remodeling of cardiac tissue [40, 42]. The changes in the heart usually primarily affect contractile activity and metabolism. Fish living in temperate climates are particularly susceptible to significant and long-term seasonal temperature changes [45–47]. Among these fish species, salmonids like rainbow trout can remain active between 4 and 24°C [42, 48]. Also, minnow fishes like zebrafish can experience a broad change in temperature of about 10°C considering fluctuations between winter and summer [49, 50]. For carp SCCs, an increase in culture temperature from 21 to 31°C caused a significant decrease in the contraction frequencies [51]. Due to their biology, fishes are provided with mechanisms to protect heart function from temperature changes. In the past, the comprehension of biological responses to lower temperatures has been the main target of experimental research. However, higher temperatures are becoming one of the main global concerns, making the biological responses to high temperatures another important field to investigate [42]. To date, several biological mechanisms in response to sharp temperature changes are already known and, in some cases, they are similar to human responses. For example, hypothermia leads to bradycardia and higher blood viscosity in both humans and fishes [41, 42]. However, some important differences between mammals and fishes can help to reach a deeper understanding of temperature change resilience strategies. For example, it has been shown that trout cardiac actin-myosin ATPase activity was more Ca^{2+} sensitive than that of rats within their respective physiological temperature [42, 52]. In addition, researchers reported that the Ca^{2+} quantity required by trout cardiac muscle to reach half-maximal tension was about one-tenth of that of rat cardiac tissue [42]. To conclude, fish *in vitro* heart

models, which can be generated from several fish species, could provide key findings into the cellular and molecular mechanisms underlying temperature-induced cardiac stress. A deeper understanding of these processes can contribute to improve health resilience in case of unfavorable environmental conditions in response to increasing global temperatures and extreme climate events.

4.2 Applied science

4.2.1 Fish cell culture in human research/pharmacology

There is a growing interest in the use of fish cells as *in vitro* model for human cardiac research because of their physiological similarity [24–26]. The electrocardiogram (ECG) of the most widely used murine cardiac model exhibits some important electrophysiological similarities to humans, including a P wave (atrial depolarization), PQ interval (conduction of impulses from the atria through AV nodes, and the His-Purkinje system to the ventricles), and the QRS complex (ventricular depolarization). However, the action potential of mouse cardiomyocytes differs significantly from that of humans [26, 53, 54], with notable differences in cardiac ion channels [24–26, 55]. One notable drawback of murine cardiac models is the absence of an ERG channel homolog (Either-a-gogo-Related-Gene channel), which limits the modeling of repolarization disturbances and the applicability of pharmacological testing. Additionally, myosin light chains (MLC2a and MLC2v) exhibit distinct development patterns in mouse cardiomyocytes. The absence of surface marker protein SIRPA in mice leads to a 10-fold higher tolerance to drugs that may be harmful to humans [34, 56].

Despite these disadvantages, fish models, such as rainbow trout and zebrafish, have gained popularity in ion channel research, as the basic properties of many ion channels are similar in fish and humans. The ERG channel present in fish models is important in drug research, especially for studying drug-induced torsades tachycardia, a specific type of ventricular arrhythmia associated with the inhibition of the rapid delayed rectifier current (I_{Kr}) and the prolongation of the QT interval on the electrocardiogram [57].

To further advance pharmacological research, the developed *in vitro* cardiac model SCC offer significant advantages, including the potential for high-throughput screening of various factors like small molecules, nucleic acids, proteins, and lipids [34]. Zebrafish research, in particular, benefits from having a fully sequenced genome, enabling the study of cardiovascular mutants to better understand the progression of cardiovascular diseases. In addition, SCCs derived from trout as well as zebrafish, hold promise for studying ion channel disruptions and identifying suitable drugs for safety screening [28, 30, 31, 34]. SCCs can be maintained in cell culture for extended periods while retaining their functionality and exhibiting contraction rates similar to the human heart [28–30]. Moreover, the pharmacological responses of L-type calcium channels using Isoproterenol and K_{ATP}-channels using Rilmakalim have been successfully demonstrated [28].

The existence of a hERG channel in these 3D SCCs further enhances their significance. In [30], extracellular field potential recordings using a multielectrode array (MEA) showed a significant prolongation of field potential duration after administration of common hERG potassium channel blockers. The addition of terfenadine at 10 μm and dofetilide at 1 μm resulted in a 2-fold and 10-fold increase in field potential durations, respectively. This response of SCCs to these drugs highlights their suitability for *in vitro* I_{Kr} assays, as required by Guideline S7B [58].

4.2.2 Fish virology

Another application of cell cultures can be found in fish virology. Fish virology is a significant research field due to the substantial economic losses caused by fish viruses in aquaculture [59]. Outbreaks of fish diseases can have severe economic and social impacts. For instance, in Chile, outbreaks of infectious salmon anemia virus (ISAV) between 2007 and 2009 resulted in losses of approximately 2 billion US dollars and 15,000 jobs in the aquaculture industry [45, 60].

The Atlantic salmon is a species known to be vulnerable to several cardiac diseases, including cardiomyopathy syndrome (CMS) caused by piscine myocarditis virus, heart and skeletal muscle inflammation (HSMI) induced by piscine reovirus, infectious salmon anemia (ISA) caused by infectious salmon anemia virus (ISAV), and pancreas disease (PD) induced by salmonid alphavirus (also known as salmon pancreas disease virus or SPDV) [33, 61–63].

SPDV has been detected in salmonids such as Atlantic salmon (*S. salar*), rainbow trout (*O. mykiss*), and brown trout (*S. trutta*) for over three decades [61, 64], and more recently, it has also been found in some non-salmonid marine species like the dab (*Limanda limanda*) [65–67]. The relatively limited host range of SPDV compared to terrestrial alphaviruses suggests that temperature sensitivity likely plays a role in virus replication [68]. SPDV is associated with two diseases impacting the salmonid aquaculture industry [69, 70]. Pancreas disease (PD) in Atlantic salmon was first recognized in 1976 and described in 1984 [71]. A similar disease in rainbow trout, initially reported in France in 1994, is called as Sleeping Disease (SD) [72]. The mortality rates associated with these diseases vary, but even the survivors' experience significant growth reduction, leading to economic losses.

The impact of fish viruses can vary depending on the specific viral strain and the type of host cells involved. Different fish species and cell types may exhibit varying susceptibility and responses to viral infections, leading to diverse outcomes in terms of disease severity and transmission dynamics. Understanding these host-virus interactions at the cellular level is crucial for effective fish virology research and the development of targeted control measures. In this research area as well, there is an effort to transition to *in vitro* models in order to reduce the use of a high number of experimental animals [45].

The SCC *in vitro* model system has therefore already been tested for use in fish virology [32, 33, 73]. Since the main focus of research here was on Atlantic salmon, the model system was renamed SCPCs (Salmon Cardiac Primary Cultures) [32]. SCPCs have proven to be valuable tools in fish virology, enabling the study of pathogens such as Salmon Pancreas Disease Virus (SPDV) and Infectious Salmon Anemia virus (ISA virus) and their effects on cardiac cells. SPDV and ISA viruses are significant pathogens that can cause severe diseases and economic losses in salmonid aquaculture. SCPCs provide a controlled and specific environment to investigate the interactions between these viruses and cardiac cells. Researchers have utilized SCPCs to examine various aspects of viral infection, including viral replication dynamics, viral entry, cellular tropism, and the subsequent molecular and cellular responses of cardiac cells. In the case of SPDV, SCPCs have shed light on the pathogenesis of the virus in cardiac tissues, including its effects on cell viability, morphology, and function [32, 33].

Moreover, SCPCs offer the advantage of representing a more biologically relevant model compared to traditional cell lines, as they maintain the characteristics and functionality of primary cardiac cells [32]. This allows for a better understanding

of the virus-host interactions within the natural cardiac environment. Additionally, SCPCs may serve as a platform for testing potential antiviral interventions, such as antiviral compounds or vaccines, to assess their efficacy in controlling viral replication and minimizing virus-induced cardiac damage in the future [33].

In conclusion, SCPCs have emerged as valuable *in vitro* models for studying SPDV, ISA virus, and other fish viral diseases. They provide insights into the molecular and cellular events underlying viral pathogenesis in cardiac tissues, aiding in the development of effective control strategies and therapeutic interventions. The utilization of SCPCs could contribute to mitigating the impact of these viral pathogens on salmonid aquaculture and improving the management of related diseases [32, 33, 73].

Conclusion

In vitro cardiomyogenic models using whole fish larvae and embryos provide a versatile platform for investigating cardiac biology and pathology. Their unique properties and applications make them valuable tools in cardiac research for both fish and humans. With further advances and refinements, these models hold great promise for unraveling the complexities of cardiac diseases and advancing therapeutic strategies, as well as examining adaptations in heart cell anatomy and physiology due to climate changes.

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Conflict of interest

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This model system has been patented and is registered under the following number: WO2011029584A1.

Acronyms and abbreviations

2D	two-dimensional
3D	three-dimensional
AP	action potential
Cx	connexin
CMS	cardiomyopathy syndrome
DMEM	Dulbecco modified eagle medium
ECG	electrocardiogram
ERG channel	either-a-gogo-related-gene channel
FP	field potential
FBS	fetal bovine serum
L-15	leibovitz's medium
HCN4	hyperpolarization-activated cyclic nucleotide-gated channel 4
HSMI	heart and skeletal muscle inflammation
Ikr	rapid delayed rectifier current
IPS	induced pluripotent stem cells
ISA	infectious salmon anemia
MLC2a and MLC2v	myosin light chains
PD	pancreas disease
SD	sleeping diseaseSD
SCC	spontaneously contracting cell aggregate
SCPC	salmon cardiac primary culturesSCPC
SIRPA	surface marker protein
SPDV	salmon pancreas disease virus
ZFHA	zebrafish heart aggregate

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