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Review

Methods for in vitro functional analysis of iPSC derived cardiomyocytes — Special focus on analyzing the mechanical beating behavior ☆

Laurila Eeva [a,*], Ahola Antti [b], Hyttinen Jari [b], Aalto-Setälä Katriina [a,c]

[a] University of Tampere, BioMediTech and School of Medicine, Tampere, Finland
[b] Tampere University of Technology, Department of Electronics and Communications Engineering, BioMediTech, Tampere, Finland
[c] Heart Hospital, Tampere University Hospital, Tampere, Finland

ABSTRACT

A rapidly increasing number of papers describing novel iPSC models for cardiac diseases are being published. To be able to understand the disease mechanisms in more detail, we should also take the full advantage of the various methods for analyzing these cell models. The traditionally and commonly used electrophysiological analysis methods have been recently accompanied by novel approaches for analyzing the mechanical beating-behavior of the cardiomyocytes. In this review, we provide first a concise overview on the methodology for cardiomyocyte functional analysis and then concentrate on the video microscopy, which provides a promise for a new faster yet reliable method for cardiomyocyte functional analysis. We also show how analysis conditions may affect the results. Development of the methodology not only serves the basic research on the disease models, but could also provide the much needed efficient early phase screening method for cardiac safety toxicology. This article is part of a Special Issue entitled: Cardiomyocyte Biology: Integration of Developmental and Environmental Cues in the Heart edited by Marcus Schaub and Hughes Abriel.

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1. Introduction

Until the latest decade, one of the main challenges in cardiac research has been the difficulty in obtaining cardiac cells for laboratory and cell culture experiments. Cardiac biopsy is a demanding and high-risk procedure, which is performed only when advantageous clinically for the patient. Furthermore, cardiomyocytes from the biopsy can be cultured in a laboratory only for a very short time period, due to rapid dedifferentiation. Invention of the protocol for differentiating functional cardiomyocytes from human pluripotent stem cells in 2003 gave one solution for the problem [1]. For the first years, the starting material for cardiac differentiation was of embryonic origin, and thus its use in research was under extensive debate. When Prof. S. Yamanaka and his research team published an article in 2007 describing how human fibroblasts can be reprogrammed to pluripotent state by using four transcription factors (OCT3/4, SOX2, KLF4, and c-MYC), the ethical concerns of the use of stem cells were overcome [2,3]. Today, generation of pluripotent stem cell derived cardiomyocytes is a routine procedure, and readily differentiated high-quality induced pluripotent stem cell derived cardiomyocytes (iPSC-CMs) are commercially available from multiple sources (Fig. 1).

Generation of iPSC-CMs has offered a great tool for the research and resulted in exponential growth of this research field. Methods for reprogramming cells to the pluripotent state, and differentiating them to functional cardiomyocytes have evolved quickly [4]. Thus, the number of iPSC based disease models for genetic cardiac diseases has increased rapidly, providing a huge amount of material to be studied. Furthermore, high-quality iPSC-CMs have become commercially available from multiple providers, enabling their use in cardiac research without the need to have iPSC laboratory. Functional in vitro analysis of cardiomyocytes is essential for both understanding the pathogenic mechanisms of cardiac disorders and performing preclinical safety studies for novel drugs. As Peters and colleagues already in 2012 stated, there is an urgent need for innovative in vitro assays with sufficient throughput and quality to enable screening of potential cardiovascular toxicity of novel potential drugs [5]. It is obvious that the methodology for functional characterization of the disease models has to develop to meet these needs. The ideal future methods are fast, yet sensitive and reliable, and enabling high throughput analysis.

Development of a new drug is a process with a high rate of failure [6, 7]. Costs of the process increase greatly especially when moving from preclinical to clinical phase, underlining the importance of thorough validation of the drug candidates in the early phases of the development pipeline. The preclinical cardiac safety studies of drug candidates
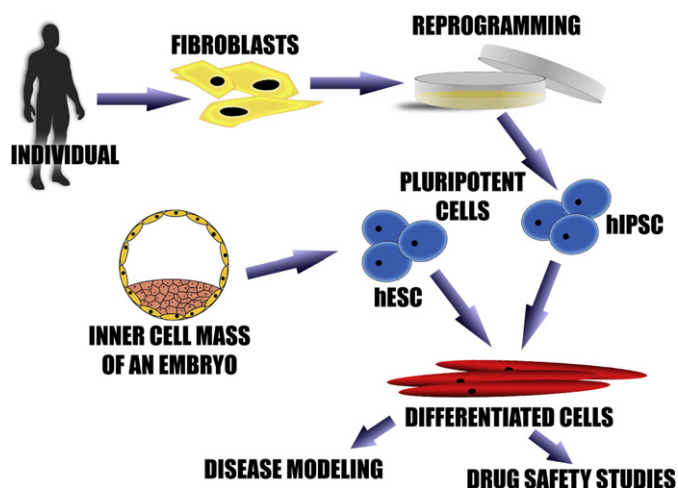
Fig. 1. Generation of pluripotent stem cell derived cardiomyocytes for cardiac research. Pluripotent stem cells can be derived directly from the inner cell mass of an embryo (human embryonic stem cells, hESC) or from differentiated cells of an individual, such as fibroblasts, via reprogramming (human induced pluripotent stem cells, hiPSC). Both of these can be differentiated to functional cardiomyocytes for different purposes.

include assays on the human ether-à-go-go related gene (hERG), a encoding for a potassium channel protein [8,9]. Inhibition of the function of this ion channel interferes with cardiac repolarization and causes prolongation of the QT interval, which may induce serious ventricular arrhythmias, such as Torsade de Pointes (TdP), and lead to sudden cardiac death [10]. This is a major cause of adverse clinical events and withdrawal of drugs from the market [11,12]. Traditionally the safety studies have been based on stable expression of the hERG gene in a non-cardiac human cell line (such as HEK293). However, these transfected cells lack all other cardiac ion channels forming in concert the proper electrical properties of cardiomyocytes and transfected cells are not an optimal platform to study human cardiotoxicity. Thus, human iPSC based models have been increasingly searched and tested for cardiac safety studies, as they provide the whole physiological expression pattern of the cardiac ion channels [13–16].

Several review articles have been published describing the progress on generating iPSC based disease models on genetic cardiac disorders [4,14,16–19]. However, much less attention has been paid on the methods actually used for characterizing functionality of these models. In this review article, we give an overview of the methodology for studying the functionality of pluripotent stem cell derived cardiomyocytes, with a special emphasis on the rapidly evolving field of assessment based on video microscopy (See Fig. 2). We also highlight the importance of optimizing and standardizing the analysis conditions, and give some examples how they might affect the results.

2. Cardiomyocyte functionality analysis

2.1. Methods for analyzing electrophysiology

2.1.1. Patch clamp

Traditionally, methods analyzing cardiomyocytes have been almost solely based on the electrophysiology of the cells. Patch clamp provides detailed data on ion currents, and is often referred to as the gold standard for cardiomyocyte analysis due to its sensitivity and ability to detect currents directly from the cell [20]. However, it is invasive as it breaks the cell membrane and requires a strong seal (giga seal), thus enabling only a short term measurement. Additionally, it is a terminal experiment leading to cell death. Patch clamp is also not an efficient screening method due to the relatively long time needed per one measurement, expensive instrumentation, and the sophisticated skills required from the personnel. Automated patch clamp of iPSC-CMs

would combine the high accuracy with a considerable throughput but has still its limitations in terms of purity and selection of the cell population to be analyzed [21–23].

2.1.2. Multielectrode arrays

Multielectrode arrays (MEAs) provide a non-invasive and non-terminal method for studying cardiomyocyte electrophysiology. Cells are plated on a dish containing electrodes on the bottom, and field potential is recorded. Field potential duration (FPD) and the field potential waveform provide information at least partially comparable to action potential data obtained from patch clamp [24,25]. MEA analysis has been used for predicting cardiotoxicity [26] and is also rather easy to scale up towards high throughput [27]. However, despite the efforts of producing a "single cell MEA", the current MEAs are able to reliably record data only from cell clusters or monolayers.

2.1.3. Fluorescent imaging

Methods based on fluorescence microscopy, such as calcium indicators or voltage-sensitive dyes, provide a non-invasive method for obtaining accurate data on intracellular ion fluctuations and voltage changes. However, the addition of a chemical indicator, which attaches to molecules inside the cell, is likely to interfere with the functioning of the cell, which must be always kept in mind when interpreting the results. Currently available calcium indicators, such as Fura-2 and Fluo-4, are toxic, and UV-light, which typically is used in detection, is also harmful for the cells [28]. Fusion genes based on green fluorescent protein (GFP) have been developed to circumvent this issue, but it might affect the folding and functioning of the proteins in the cell, and still needs the UV excitation. Traditional calcium imaging is a rather slow method, both for performing the assay itself and for data analysis, despite novel tools created for data analysis [29]. An approach towards higher throughput in calcium imaging, capable of analyzing hundreds of cells simultaneously on a multi-well plate, has been described a few years ago [30]. Voltage-sensitive dyes, such as di-4-ANEPPS, respond to changes in membrane potential by changing their fluorescence emission. They are commonly used for studying whole hearts but have their limitations in single cell and monolayer imaging due to the cytotoxicity [31,32].

2.2. Methods for analyzing mechanical beating behavior

Although in the majority of the studies the different methods for assessing only the electrophysiology of the cells are used, alternative methods would be clearly justified and even required. All genetic cardiac diseases do not alter only the electrophysiology of the cell but also the mechanical beating behavior, such as beating motion or contractile force [33–36].

2.2.1. Traction force microscopy

The force exerted by a cardiomyocyte during a contraction can be estimated with non-invasive methods using traction force microscopy. In traction force microscopy, fluorescent beads are embedded in a substrate, on which cells are cultured. The displacement of the fluorescent beads due to the cell activity can then be tracked with optical methods and cell traction force can be estimated from the displacement and the mechanical properties of the substrate [37]. The calculation methods for displacement of beads are essentially similar to the ones used in video analysis of cardiomyocytes. Hazeltine and colleagues successfully measured human pluripotent stem cell (hPSC) derived cardiomyocyte activity in 2012 [38].

2.2.2. Atomic force microscopy

Atomic force microscopy (AFM) is another method for measuring cardiomyocyte force. In AFM, a small cantilever is brought in contact with the cell being measured. While the cell beats, the vertical movement of the cell membrane is tracked producing the beating signal
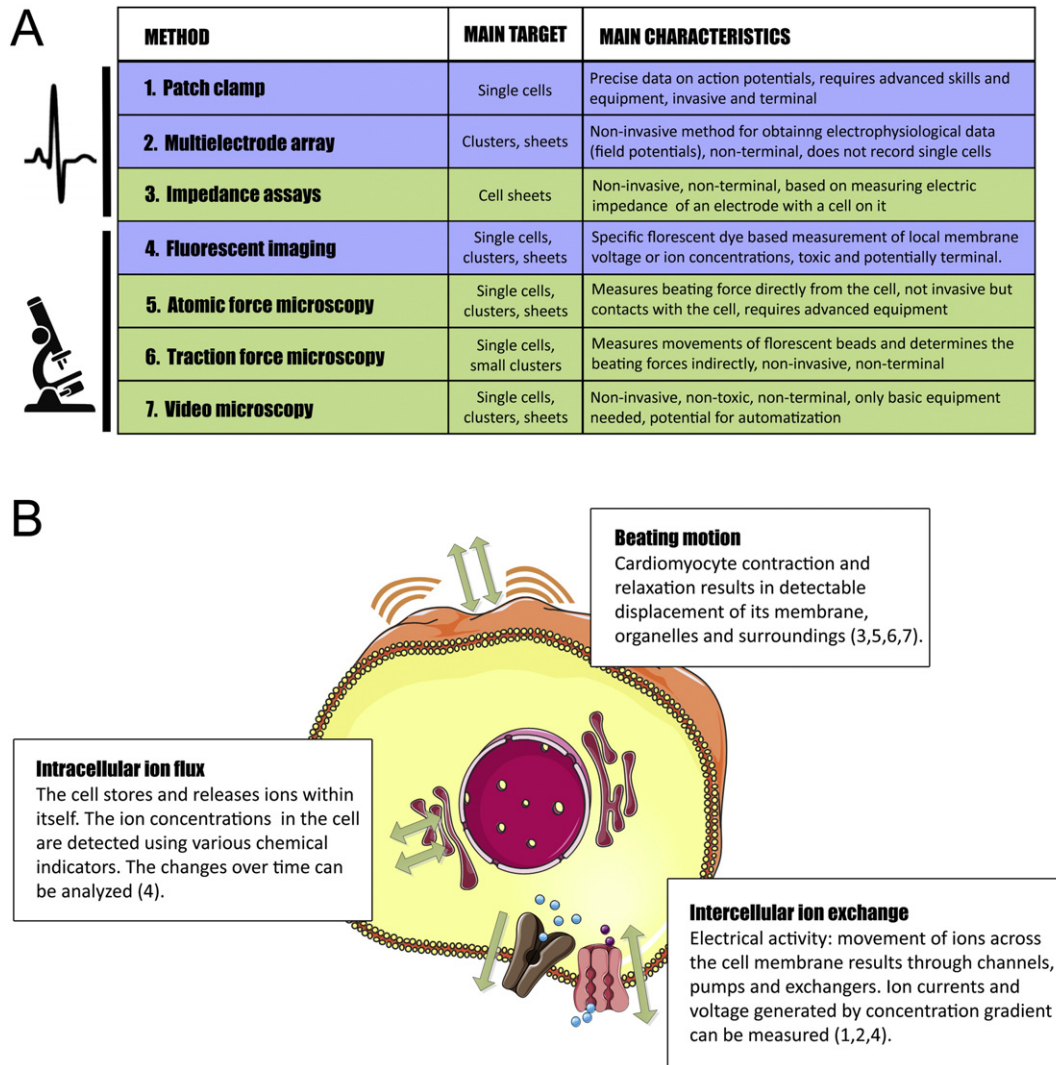
A

METHOD	MAIN TARGET	MAIN CHARACTERISTICS
1. Patch clamp	Single cells	Precise data on action potentials, requires advanced skills and equipment, invasive and terminal
2. Multielectrode array	Clusters, sheets	Non-invasive method for obtainng electrophysiological data (field potentials), non-terminal, does not record single cells
3. Impedance assays	Cell sheets	Non-invasive, non-terminal, based on measuring electric impedance of an electrode with a cell on it
4. Fluorescent imaging	Single cells, clusters, sheets	Specific florescent dye based measurement of local membrane voltage or ion concentrations, toxic and potentially terminal.
5. Atomic force microscopy	Single cells, clusters, sheets	Measures beating force directly from the cell, not invasive but contacts with the cell, requires advanced equipment
6. Traction force microscopy	Single cells, small clusters	Measures movements of florescent beads and determines the beating forces indirectly, non-invasive, non-terminal
7. Video microscopy	Single cells, clusters, sheets	Non-invasive, non-toxic, non-terminal, only basic equipment needed, potential for automatization

B

Beating motion
Cardiomyocyte contraction and relaxation results in detectable displacement of its membrane, organelles and surroundings (3,5,6,7).

Intracellular ion flux
The cell stores and releases ions within itself. The ion concentrations in the cell are detected using various chemical indicators. The changes over time can be analyzed (4).

Intercellular ion exchange
Electrical activity: movement of ions across the cell membrane results through channels, pumps and exchangers. Ion currents and voltage generated by concentration gradient can be measured (1,2,4).

Fig. 2. (A) Summary of the main characteristics of the methods currently used for iPSC-CM functionality analyses. Blue background color indicates methods assessing electrophysiology or ion fluctuations of the cell, and green color methods measuring beating force or movement of the cell. Leftmost graphic shows whether the method utilizes electrophysiology in measurement, or is based on microscopy. (B) Illustration of a cell showing different measurable properties of a cardiomyocyte along with the methods used to analyze those. Numbers refer to the methods in panel A.

[39]. hPSC derived cardiomyocyte beating characteristics have been measured with AFM in 2012 by Liu and colleagues [40].

2.2.3. Impedance assays

Assays based on cellular impedance offer a non-invasive and label-free analysis method, which has potential for higher throughput [28, 41]. Cells are seeded and allowed to attach on a multi-well cell culture plate, which contains electrodes on the bottom. When a weak electric current is applied between the electrodes, the cells attached on the electrodes impede especially the low frequency current flow as the cell membrane has high electric impedance. When the cells are moving or beating on top of the electrodes the impedance signal varies accordingly [42]. Thus this method detects the beating indirectly from the varying impedance signal based on mechanical movements of the cells. Several characteristics of the cardiomyocyte beating, including beating frequency, can be read from the output.

2.2.4. Video microscopy

In recent years, several different light microscopy methods for analyzing the mechanical beating behavior of cardiomyocytes have been published. The basic features and summary of the data obtained by these methods are summarized in chapter 5 and Table 2. All these methods are based on a video recording of a beating cell or area, which is then analyzed by different computational methods. Video based analysis lacks the information of the electrophysiology of the cell, as well as the three dimensional movement, but is a good approach for quick and yet reliable screening of the mechanical beating behavior of cardiomyocytes in high throughput.

2.3. Combining electrophysiology and beating mechanics

Although measured separately, the electrophysiology, contractile force and mechanical beating behavior of a cardiomyocyte are always connected [43–45]. To understand this in more detail, combining and comparing data from different methods have been performed in several studies [46–48]. Calcium imaging provides a solid base for studying electromechanical coupling in healthy cells. In the presented studies, calcium measurements were used for validating the mechanical movement measured with optical methods. Hayakawa and colleagues found that the maximum calcium concentration in cytosol and maximum shortening velocity occurred nearly at the same time [46]. Huebsch and colleagues, using their cardiomyocytes with genetically encoded calcium indicators found that calcium flux and contractile motion were spatially and temporally correlated [47]. The motion lagged by

several milliseconds after that rise of calcium rise and that rapid low-intensity calcium fluctuations did not cause mechanical motion. Ahola and colleagues used patch clamp as a reference study for validating the motion [48]. They found the time difference between the action potential peak and maximum displacement to be approximately 300 ms and found it to correlate with previously reported values.

3. Optimizing and standardizing the analysis conditions

Regardless of the analysis tool or method used, the importance of optimizing and standardizing of the measurement and analysis conditions cannot be overestimated. These cover both the properties of the cells to be analyzed and the external conditions before and during the experiment process.

3.1. Cardiomyocytes

Many of the methods are capable of analyzing cells in varying densities, but the outcome may vary greatly between single cells and cell sheets. Plating density has been shown to have effect on several different characteristics of cardiomyocytes, including gene expression profile and electrophysiological properties [49]. In cell clusters where other cell types than cardiomyocytes may also be present, the difference compared to single cardiomyocytes is likely to be even larger. In addition to the cell density, the age and maturation state of the cells is an important issue. Although there is no single correct answer to the questions "How old the cells should be?" and "What is mature enough?", these must be carefully assessed and standardized to obtain comparable results. Finally, the external cell culture and analysis conditions, such as culture medium, pH, gases, and temperature, must be optimized and standardized for each experiment set-up. In the next chapter, this is further studied and discussed from the perspective of video analysis methods, but the basic principles are applicable for any cardiomyocyte functionality assay.

3.2. External analysis conditions

Concerns about recording conditions, such as temperature and pH, are true for any cardiomyocyte assay. However they are taken into account if the more sophisticated experimental set-ups are used. For example, in patch clamp constant perfusion with pre-warmed buffer, typically HEPES based buffer, is used, allowing constant control of the temperature and pH. Additionally, calcium imaging is done with perfusion and heating systems. With more modern methods the external perfusion and heating have not been always clearly stated or standardized. Video based analysis of cardiomyocytes for example is a relatively easy and quick method and recording of the video only requires a good quality video camera mounted on a standard microscope. However, the recording conditions may greatly affect the beating characteristics of the cells, which then are reflected on the results. Although the equipment needed for video based analysis is not extremely costly or sophisticated, special attention should be paid on the recording conditions to obtain reliable results.

To illustrate the effect of two variables, temperature and time, on the cardiomyocyte beating, we performed a set of experiments and recorded videos while controlling these variables. UTA.04602.WT iPS cell line, generated from a healthy individual and thoroughly characterized [36], was used in all the experiments. Cardiac differentiation was done using the END-2 co-culture method [36], and videos of single beating cardiomyocytes were recorded in controlled temperature at 60 fps as previously described [50]. Nikon (Nikon, Tokyo, Japan) Eclipse TS100 microscope and Imperx (Imperx, Boca Raton, FL, USA) IGV-B1620M camera were used for all recordings.

The heating plate was set to 38 °C, resulting in a temperature of approximately 37 °C in the bottom of wells of the cell culture plate. Temperature was measured from the medium inside the well, as close

to the bottom as possible without touching the plate (temperatures shown in Fig. 3), and the reading given by the heating plate was recorded at the same time. For the experiments where the temperature was manipulated, the end point of the experiment was when the beating of the cell was either stopped (increasing the temperature) or too weak for reliable analysis (decreasing the temperature). For the constant 37 °C temperature experiment, end point was set at 60 min. Perfusion and gas exchange were not used.

First we wanted to show how increasing or decreasing the recording temperature alters the cardiomyocyte beating characteristics (Fig. 3). The cell tolerates surprisingly high temperatures without completely stopping from beating (Fig. 3A). However, at high temperature the
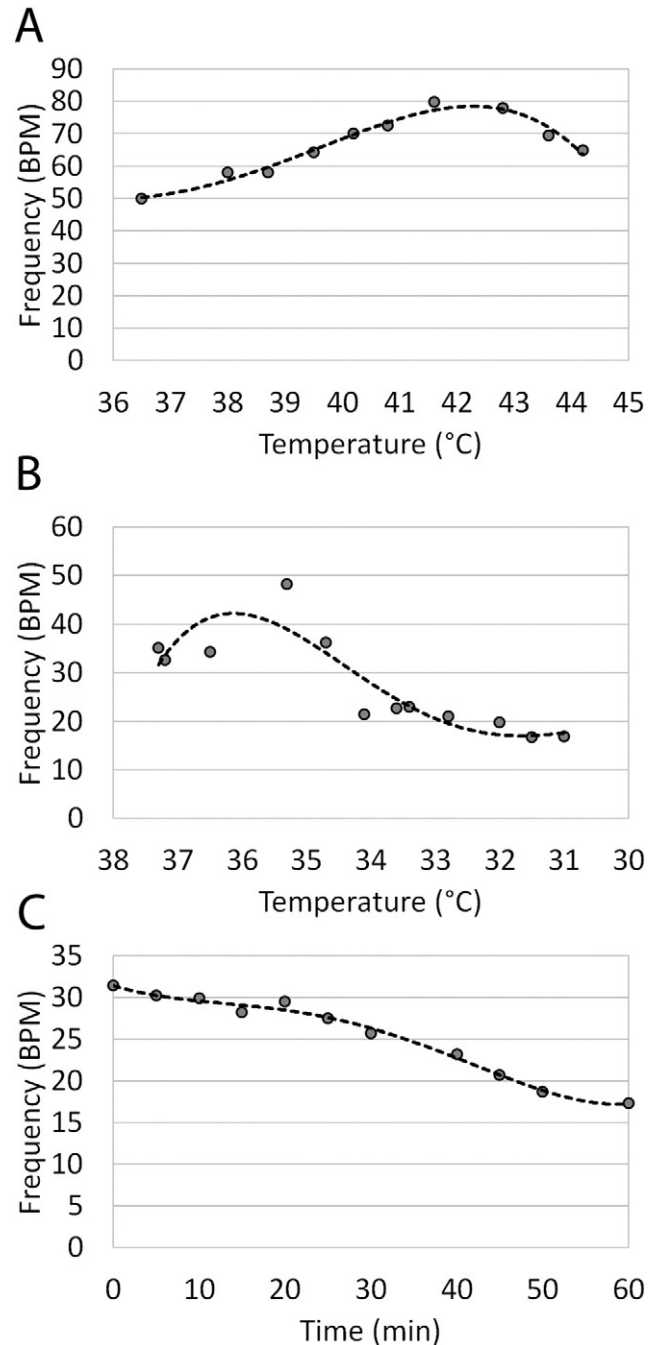

Fig. 3. Effect of temperature on the beating frequency of iPSC-CMs. Beating frequency in BPM (beats per minute) of one representative cell (total n = 6 per experiment) are shown for increasing (A) and decreasing (B) temperatures, as well as constant 37 °C temperature over a time period of 60 min (C).

beating quickly gets too faint to be analyzable without a recording set-up of greater resolution power. Lowering the temperature rather quickly leads to the cells to stop beating, underlining the importance of the use of a heating plate or perfusion (Fig. 3B). It is important to notice that even when using a heating plate on the microscope, the temperature of the cells slightly drops as soon as the cells are removed from the incubator. Fig. 3C illustrates how the beating frequency of the cells dramatically drops almost 50% in the course of time, even when the recordings are performed on a heating plate at standard temperature. This could be due to changes in other parameters in the cell culture, e.g. change in pH or O_2/CO_2, since these were not standardized in this experiment.

In the set-up for patch clamp or other electrophysiological analysis of cardiomyocytes these issues are usually well addressed. In the vast majority of the studies summarized in Table 1 in Section 4, the electrophysiological analyses were performed in temperatures between 32 and 37 °C. Our experiments underline the importance of carefully controlling the recording conditions, even though the basic set-up for recording videos of beating cardiomyocytes is rather simple. Simply the use of a standard stage incubator would solve most of the issues described above. However, experiments to verify the quality of recording conditions and set-up are certainly recommended.

4. Overview of the commonly used methods

To review the methods commonly used for the characterization of the iPSC disease models of genetic cardiac diseases, we analyzed 25 studies utilizing iPSC technology in modeling genetic cardiac disorders and summarized the characterization methods used in those (Table 1). Studies were selected from those published during the past five years. Papers in which no characterization methods were used, such as gene expression related approaches, were excluded. As

expected, the main method for studying cardiomyocyte functionality is patch clamp, which was used in all but one study. Typically the experimental approach was to use patch clamp accompanied by one additional method, usually either calcium imaging or multielectrode array. The additional methods are usually chosen by molecular defect present in the disease — for example in CPVT (catecholaminergic polymorphic ventricular tachycardia) the disease is due to disturbances in calcium transients in the cells, and thus calcium imaging is the most suitable method to describe the phenotype. In two studies, the cardiomyocytes were also analyzed based on video recordings of the cells [36,51] and one study utilized atomic force microscopy to analyze the cells [52]. In summary, the studies favor strongly the traditional methods, but are occasionally including also additional methods, if suitable in the research approach.

5. Details of the methods based on video microscopy

Video microscopy provides a non-invasive and rather easy method for cardiomyocyte beating analysis. Furthermore, it has potential for automatization and scaling up to achieve higher throughput. In recent years, a variety of methods for video microscopy based cardiomyocyte analysis has been published. Basic features of these methods are summarized in Table 2.

For any video microscopy method, the first and most important issue is the quality of the recording, which must be high enough to achieve a sufficient temporal and spatial resolution. To enable the detection of faint beating movement, the magnification and frame size must be good enough. Naturally, the definition of "good enough" is determined by the target, whether it is a single cell, a small cluster of cells or even a cell sheet or aggregate. The frame rates of the different methods seem to have more variation. High frame rate enables the detection of minor changes in the temporal scale. The average time from the start of contraction to the end of relaxation is 500–600 ms if beating rate is in the range of 40–60/min. The number of frames captured within this time period varies approximately from four (7 fps, frames per second) to 70 (125 fps), corresponding to a frame captured every 143 or 8 ms, respectively. For example when screening the safety of novel drug candidates, a 30–50 millisecond prolongation in contraction–relaxation phase could be classified as significant, the difference might become significant in detection of certain beating characteristics. However, some methods described later on in this review only aim to detect changes in the beating frequency, for which lower frame rates are sufficient [33,74]. To be able to obtain more detailed data on the beating phases, a higher frame rate is essential [48,50].

A commonly used procedure for generating beating signals from the cells is to have each video frame divided into analysis regions. A degree of similarity, or difference, between these regions in subsequent, or later, video frames is calculated. This block matching process allows the calculation of velocity and direction of the motion in each analysis window, generating a velocity vector. Based on these vectors, beating signals are calculated by calculating a measure of magnitude for each frame. Similar displacement signals can be obtained without block matching, as demonstrated by Liu and colleagues [33] and Maddah and colleagues [74]. Liu and colleagues had used principal component analysis on motion vector fields in their previous study, but in their later study, they used it on moving textures instead. Maddah and colleagues calculated the correlation of each frame with a resting-state reference frame, producing a beating signal.

Kamgoué and colleagues quantified cardiomyocyte contraction using a block-matching algorithm [75]. They used the displacement fields to calculate displacement signals. The signals were used to study single adult rat cardiomyocyte deformations and calculate shortening during contraction.

Hayakawa and colleagues described an analysis method, using a block-matching algorithm [46]. They analyzed monolayers of neonatal rat cardiomyocytes with a high 125 fps frame rate. They observed the

Table 1
Summary of the methods used for the functional characterization of iPSC models for genetic cardiac diseases in 25 studies from the past five years.

Reference	Disease	pClamp	Ca^{2+}	MEA	Others
Bellin et al. [53]	LQT2	x		x	
Caspi et al. [54]	ARVC			x	
Di Pasquale et al.[55]	CPVT	x	x		
Egashira et al. [56]	LQT1	x		x	
Fatima et al. [57]	CPVT	x	x	x	
Fatima et al. [58]	LQT3	x			
Han et al. [59]	HCM	x	x	x	
Itzhaki et al. [60]	LQT2	x		x	
Itzhaki et al. [61]	CPVT	x	x	x	
Jung et al. [62]	CPVT	x	x		
Kiviaho et al. [36]	LQT1	x	x		Video analysis
Kujala et al. [63]	CPVT	x	x		
Lahti et al. [34]	LQT2	x	x		
Lan et al. [51]	HCM	x	x		Video analysis
Lin et al. [64]	DCM	x	x		
Ma et al. [65]	LQT3	x			
Ma et al. [66]	ARVC	x	x		
Ma et al. [67]	LQT1	x			
Matsa et al. [68]	LQT2	x		x	
Mehta et al. [69]	LQT2	x		x	
Novak et al. [70]	CPVT	x	x		
Spencer et al. [71]	LQT2/3	x	x		
Sun et al. [52]	DCM	x	x		Atomic force microscopy
Terrenoire et al. [72]	LQT2	x			
Yazava et al. [73]	LQT8	x	x		

ARVC   arrhythmogenic right ventricular cardiomyopathy
BTHS   Barth syndrome
Ca^{2+} calcium imaging or similar fluorescent imaging method
CPVT   catecholaminergic polymorphic ventricular tachycardia
DCM   dilated cardiomyopathy
HCM   hypertrophic cardiomyopathy
LQT   long-QT
MEA   multielectrode array
pClamp   patch clamp

Table 2
Basic characteristics of the published methods for video microscopy based analysis of cardiomyocyte beating.

	Kamgoué et al. [75]	Hayakawa et al. [46]	Liu et al. [33]	Ahola et al. [48]	Maddah et al. [74]	Chen et al. [76]	Huebsch et al. [47]
Algorithm	Block matching	Block matching	Texture movement	Block matching	Correlation coefficient	Block matching	Block matching
Analysis area segmentation	None	None	Motion based segmentation for clustering	Beating center point based	Hierarchical clustering	None	Beating center point based
Cell culture details	Adult rat ventricular CM, single	Neonatal rat CM monolayer	SC-derived CMs, single and monolayer	hiPSC derived CMs, single	Commercial iPSC derived CMs	H9 hESC derived CMs monolayer	hiPSC derived CMs, single and clusters
Cell genotype	Not defined	Not defined	Not defined	WT (wild type)	Not defined	Not defined	WT with genetic GCaMP6f
Frame rate	Not defined	125 fps	15–30 fps	30 fps	24 fps	7 fps and 20 fps	14 fps and 100 fps
Drug experiments	None	ACh, EPI, E-4031, terfenadine, DL-sotalol, BGA, 1-heptanol	None	None	Cisapride, norepinephrine	Isoprenaline, E-4031	Isoprenaline
Reference studies	Synthetic data	None	Synthetic data	Synthetic data	None	None	None
Simultaneous studies	None	Ca²⁺ (calcium imaging)	None	Patch clamp	None	None	MEA, GCaMP6f/Ca²⁺, patch clamp
Frequency measurement	No	Yes	Yes	Yes	Yes	Yes	Yes
Beating velocity magnitude	Yes	Yes	No	No	No	Yes	Yes
Motion direction	Yes, partial	No	No	Yes	No	Yes, culture level	Yes
Beating phases	No	No	No	Yes	No	Yes, partial	No
Improvements and relevant applications		[77,78]		[36,50]	[74]	[79]	[80]

effect of various drugs and autonomous agents on the beating of the cells. To verify their findings, they performed simultaneous calcium imaging measurements. The fluorescence oscillations were consistent with the motion vector oscillations. They also performed extensive drug experiments. In their later studies [77], they used their method with hiPSC-CMs. Additionally, they used multielectrode arrays for field potential measurements and traction force microscopy for force measurements and improved their beating parameter calculation. Their method was further used for cardiomyocyte directional analysis in a clinical study on HCM (hypertrophic cardiomyopathy) cells [78].

Liu and colleagues presented an analysis method, which uses motion based deformation registration instead of block matching [33]. It uses principal component analysis on moving textures to reduce computational load. This is based on the observation that for small motion, the moving texture and motion fields are linearly related [81]. The frame rates used were 15 and 30 fps. The method allows automatic segmentation of regions based on their beating rhythm.

Ahola and colleagues introduced an analysis method for hiPSC derived cardiomyocytes [48]. Block matching using minimum quadratic difference was used for generating the vector fields for single cells. They defined beating focus points to determine beating motion direction and divided the cell further into sectors. They used 30 fps recordings. Their method was later improved by calculating correlation-based averaged waveforms, which showed the importance of using higher frame rates and the advantage of measuring signals from multiple parts of the same cell [50]. The method was used in a clinical study on LQT (long QT) cells, in which the method was improved to study the beating phases in more detail [36].

Maddah and colleagues presented an analysis pipeline, which enables automated analysis of large areas [74]. It addresses the concerns related to differences in culture density by segmenting the images into regions that beat, non-beating regions and background. Instead of commonly used block matching, their approach creates signals from calculating correlation coefficients from these regions. They used hiPSC derived cardiomyocytes in their study and recorded the videos with 24 fps.

Chen and colleagues analyzed hESC derived cardiomyocyte monolayers with their block matching algorithm [76]. They studied cardiomyocyte orientation and beating on a wrinkled substrate. With the method, they calculated motion directions on cell culture levels and determined contraction and relaxation phases. The frame rates were 7 and 20 fps. The method was later used in a platform, which automates the detection of drug effects using machine learning [79].

Huebsch and colleagues presented a method, which uses block matching [47]. They combined their analysis with GCaMP6f, a genetically encoded calcium indicator and studied single cardiomyocytes and clusters. Their method addresses some concerns of user bias by automation. They used frame rates 14 fps and 100 fps. Similarly as Ahola and colleagues [48], their method also uses beating center points for calculating velocities in different directions.

The methods described above represent the array of video based cardiomyocyte studies. The availability of human pluripotent stem cells has changed the focus of method development from mouse and rat cells to human cells. hESC and hiPSC derived cardiomyocytes do not resemble mature adult cardiomyocytes due to their less mature sarcomere structure. The difference in sarcomeric structure results in fusiform beating patterns, similar to those in neonatal rat cardiomyocytes and different from human adult cardiomyocytes. This difference has an effect on video analysis, as the directionality of the beating needs to be considered. This issue was approached by Ahola and colleagues by producing multiple beating signals from different parts of the cell [48]. However, the way these cellular subregions are defined warrants further research.

The majority of methods include a block matching algorithm, with the noted exceptions, and a measure of displacement used to produce

the signal. A commonly used measure is calculating the average beating velocity for a region of interest. However, there are some methodological problems related to this measurement. For cardiomyocyte monolayers, determining the ROI (region of interest) for calculating average displacement from cultures with multiple beating areas is not explicit. In calculating the average beating velocity, the other beating areas may have an effect on the result. Maddah and colleagues addressed this problem with their automatic analysis method [74]. For single cardiomyocytes, adhesion can have an effect on the measurements. Computing the average beating velocity may produce different results when including the non-moving areas near the cell boundaries and when only a small region from the center of the cell is considered. Further, these measurements by themselves do not provide directional information and the obtained beating signals show contraction and relaxation occurring in the same direction. For a directional signal, a frame of reference would need to be set, as was done by Ahola and colleagues and by Huebsch and colleagues [47,48].

Frame rates vary in the presented studies from 7 fps in orientation studies by Maddah and colleagues [74] to 150 fps by Tanaka and colleagues [78] for clinical studies. However, cardiomyocytes with LQT1 mutation were characterized with only 30 fps by Kiviaho and colleagues [36]. Ahola et al. considered this issue in brief when assessing the effect of frame rate on creating averaged waveform templates [50]. Due to the small sample size, the study would need to be repeated to provide more detail. Conclusive guidelines for frame rate selection have yet to be determined and will even then depend on the application and the phenotype of the cell motion.

6. Conclusions

The methodology for functional in vitro analysis of cardiomyocytes is developing rapidly. Traditional electrophysiological methods have been accompanied by novel approaches, aiming at faster and easier, yet reliable analysis of cardiomyocyte beating characteristics. In addition to the video microscopy methods, discussed in detail in this review, the entire field of optical imaging methods has advanced in the last few years. Optogenetic technologies are likely to evolve rapidly [31], novel innovations for single cell analysis can be expected in the near future [20], and the recent advances in in silico modeling offer great support for the in vitro analyses [82].

7. Future perspectives

In the human body, the cardiomyocytes are always surrounded by cells, both cardiomyocytes and other cell types. Cardiomyocyte functionality can be assessed on different levels, starting from the analysis of single cells all the way to studying the entire organ. Cell culture based methods are typically concentrating on the smaller end of the size scale (mainly single cells to small aggregates). Studying single cells is likely to reveal more detailed information of the molecular mechanisms of the disease since in a heterogeneous cluster of cells the disease phenotype might be covered by the other types of cells or mixture of different types of cardiomyocytes [53,83]. Publications from the last decade have shown great progress on simulating a much more physiological environment by creating engineered heart tissues (EHT) [84]. EHT is a structure, which consists of not only cardiomyocytes but also cardiac fibroblasts and/or endothelial cells, and provides a more complex model for studying cardiomyocyte biology. This approach has been used especially in studying myocardial injuries but has been aimed for drug safety screening as well [9,80,84]. Obviously, the findings from cell based assays cannot be directly compared to the organ level, even when performed in a tissue mimicking environment. However, some results from the cellular level have been shown to nicely correlate to the actual physiological events in the patients, such as the mutation-specific, antiarrhythmic effects of dantrolene in CPVT patients [85], demonstrating the future potential

of iPSC based disease modeling and optimizing treatments in patient-specific manner in the future.

Individualized therapy is certainly the future goal that we are aiming to reach. However, it is obvious that the routine testing of medication on patient-specific cells will require even more high throughput than the currently available tools can provide. For larger scale individualized therapy, also methods to obtain patient-specific cardiomyocytes have to be improved, e.g. direct transdifferentiation of blood or skin to cardiomyocytes, iCMs [86]. In the future, the basic clinical care of a patient with a genetic cardiac disease might include screening of compound libraries with patient-specific iPSC-CMs or induced cardiomyocytes, iCMs, to optimize the medication. The perfect future method would combine sensitivity, reliability, low cost and high throughput into one single assay. Thus, the further investigation and development of the methods described in this article, as well as novel innovative approaches are crucial for this goal to be reached.

Transparency document

The Transparency document associated with this article can be found, in online version.

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