Quantifying functional phenotypes in human pluripotent stem cell derived cardiomyocytes for disease modelling and drug discovery

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Abstract

Cardiomyocytes from human pluripotent stem cells (hPSC) are of growing interest as models to understand mechanisms underlying genetic disease, identify potential drug targets and for safety pharmacology since they may predict human relevant effects more accurately and inexpensively than animals or other cell models. Crucial to their optimal use are accurate methods to quantify cardiomyocyte phenotypes accurately and reproducibly. Here we review current methods for determining biophysical parameters of hPSC-derived cardiomyocytes (hPSC-CMs) that recapitulate disease and drug responses. Even though hPSC-CMs as currently available are immature, various biophysical methods are nevertheless already providing useful insights into the biology of the human heart and its maladies. Advantages and limitations of assays currently available looking towards applications of hPSC-CMs are described with examples of how they have been used to date. This will help guide the choice of biophysical method to characterize healthy cardiomyocytes and their pathologies *in vitro*.

Chapter 2

Measuring physiological responses of human pluripotent stem cell derived cardiomyocytes to drugs and disease

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Introduction

Almost one in three drugs are not used clinically because of side-effects on the heart[1]. Cardiotoxic drugs are often not detected in animal models or cultured cell lines expressing selected cardiac genes because their physiology differs from that of the human heart. In rodents, for example, heart rates are almost ten times faster than in humans and ion channels, most importantly the $K_v7.1$ and hERG channels, are differentially expressed[2]. Mutations in the KCNQ1 or KCNH2 genes that encode these channels and cause severe cardiac disease in humans thus have little effect in mice. It is therefore not surprising that human relevance for drug responses and modelling cardiac disease is limited.

An emerging method to model the human heart and cardiac disease is using human pluripotent stem cells (hPSCs). hPSCs can be induced to differentiate to various types of cardiomyocytes (hPSC-CMs) with high efficiency (reviewed in[3]), although most protocols to date yield hPSC-CM with a ventricular phenotype. As a result, most data reported concerns drug responses or the effect of mutations in ventricular-like cardiomyocytes. hPSCs may be either embryonic (hESCs) or 'genetically reprogrammed' adult cells called induced pluripotent stem cells (hiPSCs)[3]. hPSC-CMs are relatively immature compared with cardiomyocytes from adult heart[4] (Table 1) but nevertheless can show drug-related cardiac responses that reflect QT-prolongation and arrhythmia evident in electrocardiograms from patients[29]. hiPSC-CMs generated from patients with mutations in cardiac-relevant genes are already proving useful for understanding mechanisms of disease since they often show expected disease phenotypes[30–33]. Gene targeting is also being used to introduce cardiac disease mutations into hPSC and hiPSC from patients with mutations are being genetically "repaired" to create isogenic pairs that differ only in the genomic region of interest. This allows control over the genetic background used for study[34].

Improving the maturity of hPSC-CMs is one of the main priorities in the field. In order to assess the state of maturation as well as effects of drugs and disease, improved biophysical methods for cardiomyocyte analysis are still needed. These should be able to measure dynamic parameters such as force of contraction, calcium handling and electrical activity and preferably, should not require specialist electrophysiology or mechanobiology facilities so that they can easily be used in many laboratories. Of note though, physical stimuli, such as mechanical stress and electrical pacing, might also be necessary for cardiomyocyte maturation[35] so some specialist expertise may be required. Here, we review measurement techniques currently available for phenotyping and stimulating contractile dynamics of hPSC-CMs *in vitro* and discuss ways to modulate the physical environment to best mimic human physiological and pathological states. Cardiomyocytes resulting from different differentiation protocols can vary in their stage of maturation but in general ion currents, sarcomeric structure and calcium handling stabilize 20 to 30 days after initiation of differentiation. The methods described in this review have generally been used once this stability has been established. We provide examples of how these approaches have been used on healthy and diseased hPSC-CMs.

Table 1: Biophysical parameters of human stem cell derived ventricular cardiomyocytes during development and primary cardiomyocytes.

	hPSC-CM				hPSC-EHT		Primary cardiomyocytes	
	early		late		late		adult	
Biophysical parameter	**value**	**ref**	**value**	**ref**	**value**	**ref**	**value**	**ref**
Force of contraction (mN/mm2)[a]	0.3	5	0.5	6	4.4	7	51	8
Cell aspect ratio (length-to-width)	01:02	9	01:04	9	unknown		01:07	10
Sarcomeric organization	disorganized	9	organized	9	highly organized	11	highly organized	
Sarcomeric distance (µm)	1.65	12	1.81	12	unknown		2.15	8
Conduction velocity (cm/s)	< 2	13	< 20	14	<26	14	100	15
Multinucleated cells (%)	5	16	20	16	25	17	26	18
Mitochondria-to-cell volume ratio	0.06	19	0.09	19	unknown		0.3	20
Resting membrane potential (mV)	-50	21	-73.5	22	- 50[b]	11	-81.8	23
Voltage upstroke velocity (V/s)	< 9	21	26.2	22	8[b]	11	215	23
Calcium Transient Duration (ms)	unknown		< 370 (CaDT90)	24	< 375 (CaDT80)	25	~ 300 (CaDT90)	15
Calcium Transient Rise time (ms) (80%)	unknown		> 10	26	< 100	25	unknown	
Calcium Transient Decay (ms) (80%)	unknown		> 50	26	< 150	25	unknown	
ATP level	<2000[c] (lum/#cell)	27	<3000[c] (lum/#cell)	27	unknown		5.69 (mmol/kg weight)	28

[a] Some differences may be explained by a difference in measured contraction phase (isotonic vs. isometric) or method (beads vs. posts).
[b] Measured on dissociated cardiomyocytes from EHTs.
[c] Relative values.
Abbreviations: ATP, Adenosine triphosphate; EHT, engineered heart tissue; hPSC, human pluripotent stem cells; hPSC-CM,human pluripotent stem cell-derived cardiomyocytes.

Finally, assuming limitations with respect to maturity of hPSC-CM *in vitro* and assay methods are overcome, we look forward towards future use of these human models in drug discovery and clinical translation.

Characterizing dynamics of hPSC-CMs

Action potential

The cardiac action potential (AP) is shaped by tightly regulated ion currents, most importantly the Na^+, Ca^{2+} and K^+ current. hPSC-CMs are considered electrically immature: in contrast to their adult counterparts ventricular hPSC-CMs show spontaneous beating and more depolarized resting membrane potentials (Table 1) caused by high pacemaker current (I_f) and low inwardly rectifying K^+ current (I_{K1}), respectively. Low expression of Na^+ channels causes slow upstroke velocity in the AP (Table 1) compared to adult ventricular cardiomyocytes; recent *in silico* analysis based on previously published hiPSC-CM electrophysiological data shows an increased sensitivity to L-type Ca^{2+} block due to overexpression of the Na^+/Ca^{2+} exchanger[36]. For a complete overview of ion current differences between hPSC derived and adult cardiac cells, we refer the reader to[22]. AP characteristics are distinct for each cardiomyocyte subtype. The subtype formed during differentiation can be directed by timed addition of cytokines and hormones: retinoic acid for example directs formation of atrial-like cardiomyocytes[37] and a Smoothened Agonist (SAG) in combination with insulin-like growth factor-1 (IGF-1) induces pacemaker-like cardiomyocytes[6]. Three experimental approaches are available to measure ion currents and APs in hPSC-CMs: patch clamp electrophysiology, voltage sensitive sensors and multi electrode arrays (MEAs). Each has its own advantages and disadvantages.

Patch-clamp electrophysiology allows precise measurements of ion currents and membrane potentials by voltage- and current clamping, respectively. It can be used to determine the identity of the cardiac subtype and specific drug responses. For example, atrial and ventricular cells from hPSC and their differential responses to the atrial specific drugs vernakalant and XEN-D0101 27 to 30 days after initiation of differentiation were evident in patch clamp assays[37]. Since every cardiomyocyte is "clamped" individually and a well-sealed pipette-membrane interface is essential, the procedure is labour intensive and dependent on operator skills. To address this, Scheel et al. used automated whole-cell patch clamp (CytoPatch 2) and measured responses to nifedipine, cisapride and TTX in commercially-supplied ventricular hiPSC-CMs that would be expected from clinical data and conventional patch clamp[38]. Similarly, using the IonWorks Barracuda system, a group of 353 compounds that included known hERG channel blockers, largely gave predicted pharmacological responses in HEK293 and CHO-K1 cells expressing hERG channels[39]. However, these automated whole-cell patch clamp systems measure APs by patching the cardiomyocytes whilst they are in suspension and not attached to a substrate. Under these conditions, cardiomyocyte lifespan is limited so that experiments need to be complete within a short time window. Automated patch clamp is designed for high throughput phenotyping of many cells under different conditions but manual patch clamp is at present more accurate.

A less invasive and labour intensive method is based on electrochromic fluorescent voltage-sensitive dyes (VSDs)[24], such as di-4-ANEPPS. VSDs are fast-response fluorescent probes that intercalate between lipid bilayers; they respond to changes in electrical field by fluctuations in fluorescence intensity that can be recorded optically. In standard (low-speed) optical systems the major limitation is the sampling frequency of the optical recorder. The upstroke velocity, the fastest kinetic parameter of the AP, is about 285 V/s in adult ventricular cardiomyocytes[23,40] causing a positive change in the membrane potential of about 130 mV. The minimum sample frequency of this near linear transient is around 8.8 kHz.hPSC-CMs have smaller upstroke velocities (10-100V/s) so that such high frequencies might not be required. Genetically encoded fluorescent voltage sensors, first used to map neural signalling pathways[41], are also potentially useful optical alternatives. Although they have improved since first introduced and have been stably expressed in hPSC-CMs, they have longer response times than VSDs, limiting their utility (Supplementary Table 1).

Microelectrode arrays (MEAs) are medium throughput, non-invasive alternatives that requires less operator training and specialized equipment compared to patch clamp. These are glass culture substrates containing electrodes that measure external electrical activity by transducing the extracellular ion flux into an electrical current readout of field potential. Several studies have shown that near-equivalents of QT-prolongation and changes in upstroke velocities can be measured as external field potentials. hESC-CMs exposed to compounds affecting AP duration[29] for example, or hiPSC-CMs from long QT patients with KCNQ1 mutations showed significant responses in MEA-recordings 20 to 30 days after initiation of differentiation[42]. Spira et al. recently manufactured MEAs with sharp electrodes that can penetrate the cell membrane and directly access the cytosol, much like patch-clamp[43]; however, variation in access resistance of the electrode and poor seal formation, as in the automated systems, limited their use as an alternative to conventional patch-clamp.

VSDs and MEAs have an additional advantage: cardiac conduction velocities can be measured as the wave propagation velocity in hPSC-CM monolayer cultures; this can reveal defective electrical conduction in the heart. Thompson et al. for example used VSDs to show improved AP conduction after engrafting ventricular-like hESC-CMs in a neonatal rat ventricular-cell model of arrhythmogenic cardiac tissue[44].

Calcium flux

The inflow of Ca^{2+} ions during the cardiac AP affects the AP itself and the shape of the contraction transient in hPSC-CMs. The kinetics of the Ca^{2+} flux provide information on calcium handling which is carefully orchestrated by the L-type channels, RYR2-channels and sarco/endoplasmic reticulum Ca^{2+}-ATPase (SERCA); this is of utmost importance in revealing disease phenotypes[32] and in predicting drug responses, toxicity and cardiac safety[45,46]. Fluorescent probes that bind Ca^{2+} are the method of choice for calcium transient measurements (reviewed in[47]). Gepstein et al. for example used Fluo-4 to investigate calcium handling by hESC-CMs. He showed an increase in sarcoplasmic reticulum calcium load as a function of time *in vitro* and predicted Ca^{2+} storage release in response to caffeine and ryanodine[26].

In general, fluorescent calcium sensitive detectors (CSDs) are categorized as ratiometric or non-ratiometric, the most important difference being their ability to measure absolute versus relative concentrations of Ca^{2+}. Ratiometric dyes allow bound and unbound dye molecules to be determined; from these values the absolute concentration of Ca^{2+} can be calculated[47]. This ratio of bound- to unbound dye molecules is not known when using non-ratiometric CSDs; whilst changes in fluorescence intensity reflect transient changes in cytosolic Ca^{2+} concentration these values cannot be translated to the absolute Ca^{2+} concentration without calibration. Additionally, there is inter-assay variability since the intracellular dye concentration is dependent on loading time, temperature, cell permeability and cell access (e.g. cell cluster size) rather than the loading concentration[47]. This makes keeping the intracellular dye concentration constant between experiments very difficult. The change in Ca^{2+} concentration is often calculated as a pseudo-ratio dF/F[48]. Although this pseudo-ratio adequately relates the change in signal to the change in Ca^{2+} concentration, it is sometimes incorrectly interpreted since the maximum value depends on loading conditions. Relative changes are best calculated and compared within a zero-to-one scale defined by the baseline conditions, eliminating confusing values for the maximum amplitude.

In hPSC-CMs, the relative change in Ca^{2+} response resulting from disease mutations or drugs is often more important than absolute levels. For example, Kosmidis et al. recently showed Ca^{2+} handling increased relatively in response to the synthetic glucocorticoid dexamethasone in ventricular hESC-CMs 28 to 30 days after initiation of differentiation[21]. It has also been shown that reduced caffeine-induced NCX currents in hiPSC-CMs from patients with catecholaminergic polymorphic ventricular tachycardia (CPVT) were evident as similar decreases of fluorescence intensity of the CSD[49]. A recent review on calcium signalling in hPSC-CMs[50] concluded that well-characterized hPSC-CMs are often reliable models for human cardiomyocyte calcium handling, since all calcium parameters of cardiac signalling are present and functional: calcium current induced Ca^{2+} release, Ca^{2+} sparks, NCX currents, SERCA2a and ß-adrenergic regulation. We would like to add that although CSDs are among the most valuable tools for evaluating hPSC-CMs, caution is needed in interpreting data since not all calcium regulating organelles are necessarily fully developed *in vitro* (e.g. t-tubules) possibly impacting conclusions.

Genetically encoded CSDs are relatively new; they were initially developed for neuroscience applications *in vivo* and have outperformed synthetic Ca^{2+} dyes in their sensitivity[51]. However, as for the genetically-encoded VSDs, slow on-and-off kinetics currently limit their use for cardiac mapping[52]. Both ratiometric and non-ratiometric detectors can easily be transduced and stably expressed in hPSC-CMs but variability in expression levels introduces similar inter-assay variation as the loading conditions of dyes.

Force of contraction

Heart failure is associated with decreased contraction force or a change in its kinetics[53]. Bray et al. showed that myocyte geometry dictates sarcomeric alignment[54]; in ventricular hPSC-CMs this was optimal at width-to-length (or "aspect") ratios of 1:7 and that it determined the net force of contraction[55]. The maximum contraction

Table 2: Qualitative comparison of measurement methods for hPSC-CMs phenotyping.

Relative scores of the pros and cons in measuring important features of hPSC-CM. The resolution, speed, ability to multiplex with other techniques, (non)-invasiveness of the technique , its accuracy (how close the output value is to the actual value), precision (how great the spread in values) and the ease of setting up the technique are given relative values (++ high to - low) to guide the choice made for phenotyping.

Measurement parameter	Methods	Tissue type	Resolution	Speed	Multiplexing	Non-invasiveness	Accuracy	Precision	Easy to set up	High throughput
Force of contraction	Beads	Single cell	+	+	++	++	+	++	--	+
Force of contraction	Flexible poles	EHT; single cell	+/-	+	-	++	+	+	--	++
Force of contraction	Skinned myocyte measurement	Single cell	++	++	+/-	--	++	++	+/-	--
Calcium transient	Non-Ratiometric dye	Any	++	+	+	+/-	+	++	++	+
Calcium transient	Ratiometric dye	Any	++	+	-	+/-	++	++	+	+
Calcium transient	Non-ratiometric protein construct	Any	++	--	+	+/-	--	-	-	++
Action potential	Voltage sensitive dye	Any	++	+	++	+	+/-	++	+	+
Action potential	Voltage sensitive protein construct	Any	++	--	++	+	--	-	-	++
Action potential	Patch clamp	Single Cell	++	++	-	--	++	++	-	--
Field potential	MEA	Monolayer; aggregates	++	++	++	+/-	++	+	+	+
Conduction velocity	MEA	Monolayer	++	++	++	+/-	++	+	+	+
Conduction velocity	Voltage sensitive dye	Any	++	+	++	+	+/-	++	+	+
ATP level	ATP fluorescent sensor*	Any	++	-	+/-	+	+	++	+/-	+
ATP level	Extracellular flux analyser	Monolayer	++	-	+	--	+	++	+/-	++
Sarcomeric organization	Protein expression construct	Any	+/-	+/-	++	++	+/-	+/-	-	+

* This technique has not yet been used on hPSC-CMs.

forces reported for hPSC-CMs are always an order of magnitude lower than for adult cardiomyocytes (see Table 1). Contraction of cardiac muscle is characterized by isotonic and isometric phases, where tension is developed with- and without the cell being able to shorten, respectively e.g. in free space or attached. Generally, the isotonic phase of hPSC-CM contraction has been assessed most often[5] and although techniques have been developed that assess the isometric phase of single cardiomyocytes[56], they have not yet been used on hPSC-CMs.

Measurements of isotonic tension on hPSC-CMs have been done on single cells, or on 2D and 3D multicellular cultures. Rape et al. developed polyacrylamide (PA)-coated coverslips containing fluorescent beads, with 20 μm wide microcontact-printed gelatine lines to guide orientation and elongation of single cells[57]. We used this technique (Figure 1A and D) recently to show that a commercially available culture medium for cardiomyocyte maturation increased contraction force of ventricular hESC-CM 33 days after initiation of differentiation compared to human fetal ventricular cardiomyocytes[5]. Another study by Rodriguez et al. using an array of small polydimethylsiloxane (PDMS) microposts showed higher contractile velocities in single ventricular hiPSC-CMs on a surface coated with laminin compared to fibronectin and collagen IV[17]. Of note though, the position in the z-axis of the post to which the cardiomyocyte attached affected its deflection so that correction for this underestimation is needed. Also, since the stiffness of the substrate on which hPSC-CMs are plated influences the force they generate[58], direct comparison of absolute values from different assays (i.e. with different substrates) is not meaningful.

Muscular thin films (MTFs) or bio-hybrid films are 2D constructs first developed for use with neonatal rat ventricular cardiomyocytes by Feinberg et al. (Figure 1B). They showed that greater tissue alignment resulted in a higher peak systolic stress[59]. Since then, MTFs have been proposed as *in vitro* contractility assays for drug screening and disease modelling[60]. However, Hinson et al. recently showed that 2D constructs do not recapitulate the structured architecture of native cardiac tissue well enough to capture the diseased phenotype in MTFs from patient hiPSC-CMs with a mutation in the sarcomeric protein titin. In contrast, 3D cardiac tissues generated from the same patient-derived iPSC line clearly showed reduced contraction[61].

Other approaches for measuring force of contraction include Engineered Heart Tissue (EHTs), cardiac microtissues (CMTs) and cardiac microwires (CMWs). These are millimetre-sized biomaterial 3D structures seeded with cardiomyocytes *in vitro,* which mimic cardiac myobundles (Figure 1C). EHTs are attached to PDMS posts and their deflection is directly related to the force exerted by the cell. hPSC-CMs incorporated in EHTs have been reported to be more mature than conventional 2D cultures, exhibiting highly organized sarcomeres, myocyte elongation and multinucleation (Table 1). For this reason and because of cell population heterogeneity that includes many fibroblasts, EHTs are strong candidates as models of mature functional myocardium. EHTs induce clinically relevant drug responses, such as the positive inotropic effects of isoprenaline, in ventricular hiPSC-CMs from day 25 on after initiation of differentiation[25]. Despite their exceptional myocardial maturity, the immediate utility of hPSC-CM-EHTs in high-throughput screening is limited by the large numbers of cells they require, drug

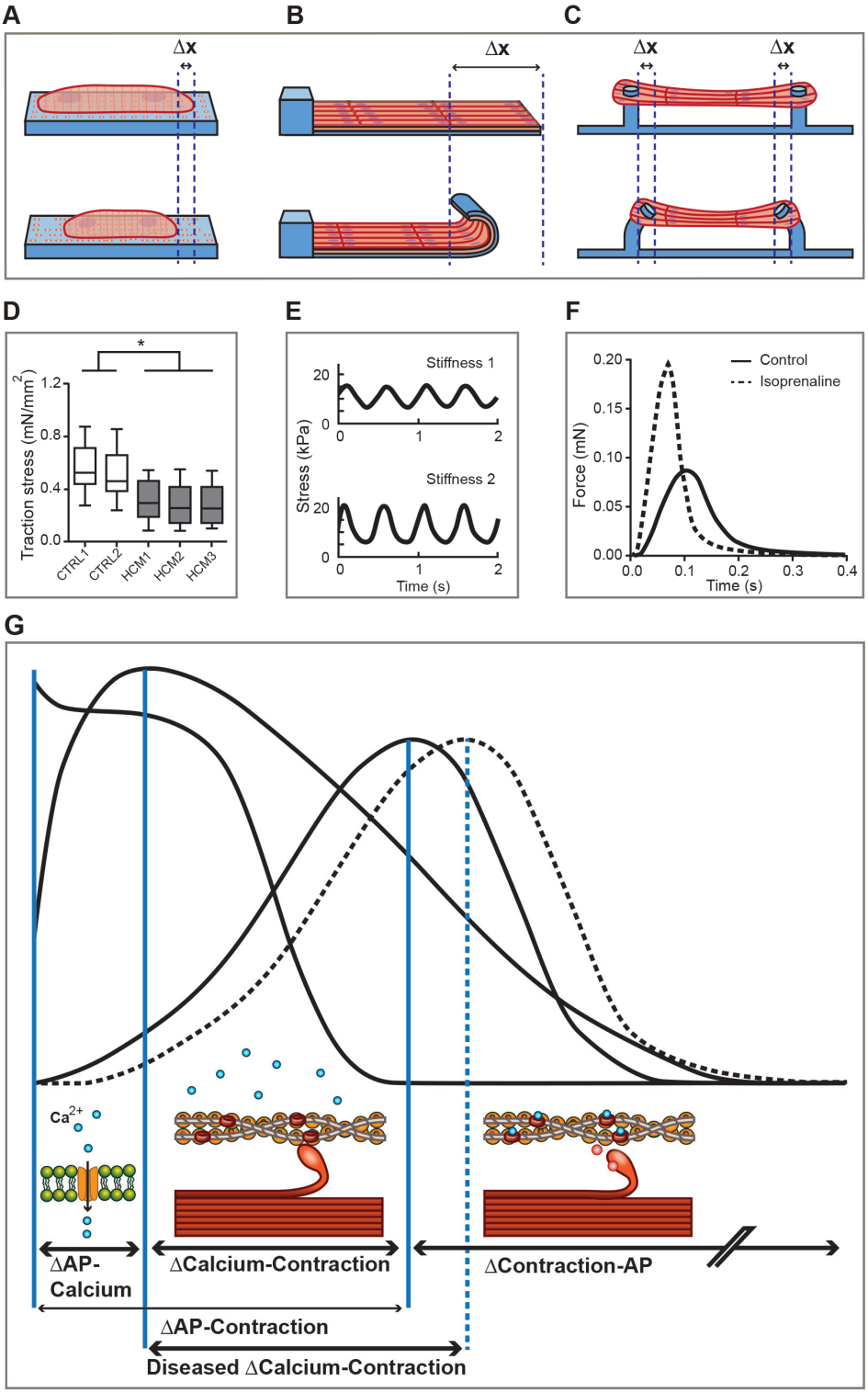
penetration and challenges with imaging. However, they are particularly amenable to analysis using classical physiology techniques such as "skinning" often used on explanted primary heart tissue, in which cytoplasm and membranes are removed leaving just the sarcomeric structures intact for force measurement[8].

Apart from measuring mechanical displacement, the kinetics of contraction force can also be determined from sarcomere movement, motion vector analysis and (as a surrogate) impedance measurements. During contraction, the sarcomere shortens and the distance between Z-disks is reduced. This can be measured in hiPSC-CMs as a change in sarcomeric distance which can be monitored using genetically encoded fluorescent alpha-actinin reporters built into hiPSC or live staining to visualize sarcomeres directly[55]. However, imaging systems with high spatial (~200 nm) and temporal (~30 ms) resolution are required to assess kinetics accurately. Motion vector analysis by contrast does not require such high spatial resolution, ectopic reporters or staining methods. It uses transmitted light microscopy to calculate the kinetics of contraction through, for example, edge detection algorithms or, more recently, an algorithm evaluating frame-to-frame similarities[62]. After taking substrate mechanics into account, this technique compares well with other measurement techniques, illustrating its utility[62]. Impedance measurements have also been used as readouts of contractile kinetics[63], but to date these have not been compared quantitatively with established technologies. Impedance measurement detects alterations in resistance of cardiomyocytes as a function of shape changes during contraction. xCELLigence and CardioExcyte96 are among the most widely used devices for measuring impedance. Results are encouraging[64] but require further validation.

Multiplexed measurements

More valuable are attempts to measure multiple dynamic parameters in hPSC-CMs simultaneously, since their temporal correlation can provide crucial insights into drug responses or disease mechanisms. For example: diseased hiPSC-CMs might have a genetically defective contractile apparatus, which delays the interval between the calcium flux and the contraction transient and/or alters drug response (Figure 1G).

The integration of multiple standard optical assays is an approach now being developed commercially for hPSC-CMs. Q-State Biosciences (Cambridge, Massachusetts) for example is offering a platform to analyse calcium flux and electrical activity simultaneously, although initially on neural cells. Relatively slow genetically-encoded dyes may limit use for hPSC-CMs at present but this will undoubtedly improve (Table 2). Clyde Biosciences (Glasgow) measure calcium, contraction and AP in the same cells sequentially as a service but at present without temporal correlation. Drawbacks of these multiplexed measurements include their invasiveness, light-induced generation of reactive oxygen species[65] and chemical interactions of the surface of the substrate with the cardiomyocytes. As an example of invasiveness, the chemical interaction of the calcium indicator with free cytosolic calcium ions will reduce the number of free calcium ions available for the contractile apparatus. When the force of contraction is measured in the presence of a calcium indicator, the reduction in free cytosolic Ca^{2+} will cause a significantly lower outcome in force. Since interactions between the techniques


A
B
C
Δx
Δx
Δx
Δx
D
E
F
*
Traction stress (mN/mm²)
1.2
0.8
0.4
0.0
CTRL1
CTRL2
HCM1
HCM2
HCM3
Stiffness 1
Stiffness 2
Stress (kPa)
20
0
0
1
2
Time (s)
Control
Isoprenaline
Force (mN)
0.20
0.15
0.10
0.05
0.00
0.0
0.1
0.2
0.3
0.4
Time (s)
G
Ca2+
ΔAP-
Calcium
ΔCalcium-Contraction
ΔContraction-AP
ΔAP-Contraction
Diseased ΔCalcium-Contraction

used for measurement can impact their outcome, Table 3 summarizes and compares the most important features of dynamic measurement techniques qualitatively and scores their applicability in multiplexed systems. This table can be used as a starting point to select combinations of bioassays for hPSC-CMs that are compatible because they do not show mutual interference.

Table 3: State of the art response times of fluorescent calcium and voltage sensors.

Sensor	Response time	Ref
VSDs (ANNINE-6plus)	~ 10 fs	77
CSDs (Fluo-4)	~ 1 ms	78
Genetically encoded voltage probe (Ace1Q-mNeon)	< 1 ms	79
Genetically encoded calcium probe (GCaMP6f)	> 10 ms	51

Figure 1: Methods for measuring contraction force in hPSC-CMs in single cell, 2D and 3D constructs.

hPSC-CM cultured as single cells, 2D muscular thin film (MTF) or 3D engineered heart tissue (EHT) and corresponding methods for contraction force measurements. In (A) fluorescent beads are present in the elastic substrate, their displacement caused by the contraction of a single hPSC-CM is measured and used to calculate the resulting stress. In (D) this method is used to show a difference in maximum force of contraction in hiPSC-CMs with MYBPC3 mutations (HCM1, HCM2 and HCM3) compared to control (CTRL1, CTRL2) (adapted from75). Similarly, the deflection of MTFs can be used to calculate the force (B). The stress generated on different substrates with varying elasticity (E) (adapted from76) can be used to calculate the actual force of contraction. EHTs are fabricated around silicon rubber posts that deflect during contraction (C) and can be related to the force of contraction. This has been used to show the change in force of contraction in response to isoprenaline (F) (adapted from25).

In (G) the solid traces show an example of simultaneous measurement of action potential (AP), calcium flux (Ca^{2+}) and contraction, which allows correlation between these biophysical parameters in time (vertical blue lines) to gain mechanistic insights in diseases and drug responses. Aspects of disease or drug responses reflected in the traces that might be compromised in hPSC-CMs are shown below: influx of calcium ions (blue circles) to the cytosol, binding of the myosin head to the actin filament and the release of the myosin head by binding of calcium to the filament. The dotted trace indicates an example of a diseased phenotype: if the calcium binding site on the filament is compromised in a diseased hPSC-CM one would expect the delay between Ca^{2+} and contraction to be longer, while the delay between AP and Ca^{2+} remains unaffected.

Applying biophysical stimuli

hPSC-CMs not only exert stress, and strain during contraction but they can also respond to imposed biophysical stimuli.

Mechanical stimulation

Kensah et al. showed that increased static stress enhances sarcomere alignment, cell-to-cell coupling and force of contraction in ventricular hPSC-CMs[7]. Salameh et al. reported on using "cyclic mechanical stretch" (CMS) to induce alignment[66]; CMS has also been shown to increase intercellular coupling between cardiomyocytes[67]. In addition, manipulating the magnitude or frequency of mechanical stress might also be used to reveal disease phenotypes *in vitro* that would not otherwise be evident; an example would be hypertrophic cardiomyopathy which *in vivo* can manifest as sudden death during exercise. Force transducers (as in the Flexcell Tension FX-series) or an oscillating vacuum underneath a thin film substrate[68] can be used to induce (uniaxial) stress on substrates. Although PDMS is often the material of choice we, and others[69], have encountered cell detachment during stretching due to the hydrophobic character of the material. Special coatings are currently being developed to solve this problem.

Electrical field and optical stimulation

Mature ventricular cardiomyocytes do not generate spontaneous APs but rely on pacemaker activity for contraction that is regulated by the sinus atrial node (SAN). Since hPSC-CMs are often cultured as isolated ventricular cell populations without SAN cells, electrical stimulation might be key to inducing maturation *in vitro*[70]. In addition, congestive heart failure (CHF) can be induced *in vivo* by forcibly "pacing" the heart at increased frequencies (reviewed by Moe et al.[71]) and may also be induced in (diseased) hiPSC-CMs *in vitro*. Such frequency-dependent behaviour emphasizes the importance of absolute beating frequency control in ventricular hPSC-CMs, either to exclude inter-assay variation or to investigate pacing-induced effects.

Exposing cardiomyocytes to short electric fields of 0.5 to 10 ms, causes the membrane to depolarize and initiates an AP. Long-term pacing experiments have however been limited by electrolysis at the culture medium-electrode interface. This can be reduced by perfusion or frequently refreshing medium but the issue is circumvented using optogenetics.

One optogenetic approach is based on channelrhodopsin-2 (ChR2), a light gated sodium ion channel originally found in algae to enable light induced movement. Nagel et al. showed that, when expressed in mammalian cells, the influx of Na^+ initiates the action potential cascade directly[72]. Zhuge et al. demonstrated that ChR2 can be used to depolarize hESC-CMs *in vitro*[73]. However, hPSC-CM cultures *in vitro* often lack adequate concentrations of retinal – essential for the conformational change under the influence of blue light – so that ChR2 channels do not respond; this can be solved by adding retinal to the medium[74].

Outlook

Rapid technological advances in human pluripotent stem cell biology over the past 5 years are poised to transform disease modelling and drug discovery for the heart. hPSC-CMs capture genetic variation among healthy individuals and patients with cardiac disease. The development of CRISPR/CAS9 for efficient genetic modification of hPSC now allows the generation of isogenic pairs – or even series – of diseased and healthy hPSC-CMs, differing only in the gene of interest[42]. Shortcomings remain, the most significant being hPSC-CM maturity: they are not yet adult cardiomyocyte mimics. Improved and defined culture conditions, heterogeneous cell populations and 3D tissue engineering are, however, contributing to finding solutions and in at least two cases – hypertrophic myopathy caused by mutations in MYBC3[75] and titin[61] – these have been crucial in revealing the disease phenotype. Further improvements in (bio) physical conditions may be expected to allow more cardiac phenotypes in hPSC-CMs to be revealed with greater differences in experimental and control values.

Improved methods to assess responses and drivers of physiological changes in hPSC-CMs are still essential to derive full benefit from the opportunities presented by patient cells. New multiplexed systems will soon allow temporal correlation between multiple cardiac features in various contexts of cell population composition, tissue architecture and culture media so that these models accurately mimic physiological and/or pathological characteristics of cardiac tissue. As these models become more complex, they are likely to require more sophisticated measurement techniques as readouts. For example, all cell types in the heart, not just those that are contractile, could be assessed separately by introducing different genetically encoded fluorescent probes into cell type specific loci. Combined with multiplexed optical systems it would then be possible to assess and correlate the different cell type responses, providing insight into their interactions and identifying the major cellular culprit in cardiac disease. The whole range of single cell and multicellular heterogeneous cell population assays will likely be necessary depending on specific requirements for research on underlying disease mechanisms to drug discovery and repurposing.

In order for Pharma to adopt hPSC-CM models in their drug development trajectory, these integrated measurement techniques will have to be scalable and high-throughput. This will reduce the costs and make the "price per data point" acceptable in a business model. Multidisciplinary collaboration between engineers, stem cell biologists, physicists, materials scientists is the way forward in implementing hPSC in the next generation of cardiac drug discovery and disease research.

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