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Authors

Blair, Cheavar A

Pruitt, Beth L

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Mechanobiology Assays with Applications in Cardiomyocyte Biology and Cardiotoxicity

*Cheavar A. Blair and Beth L. Pruitt**

Cardiomyocytes are the motor units that drive the contraction and relaxation of the heart. Traditionally, testing of drugs for cardiotoxic effects has relied on primary cardiomyocytes from animal models and focused on short-term, electrophysiological, and arrhythmogenic effects. However, primary cardiomyocytes present challenges arising from their limited viability in culture, and tissue from animal models suffers from a mismatch in their physiology to that of human heart muscle. Human-induced pluripotent stem cell-derived cardiomyocytes (hiPSC-CMs) can address these challenges. They also offer the potential to study not only electrophysiological effects but also changes in cardiomyocyte contractile and mechanical function in response to cardiotoxic drugs. With growing recognition of the long-term cardiotoxic effects of some drugs on subcellular structure and function, there is increasing interest in using hiPSC-CMs for in vitro cardiotoxicity studies. This review provides a brief overview of techniques that can be used to quantify changes in the active force that cardiomyocytes generate and variations in their inherent stiffness in response to cardiotoxic drugs. It concludes by discussing the application of these tools in understanding how cardiotoxic drugs directly impact the mechanobiology of cardiomyocytes and how cardiomyocytes sense and respond to mechanical load at the cellular level.

1. Introduction

Cardiomyocytes (CMs) are the contractile motors of the heart that generate the force required to pump blood into the pulmonary and systemic circulations. Under normal conditions, these cells are dynamic and have an intrinsic ability to alter the amount of force they produce in response to changes in hemodynamic pressures and adrenergic stimulation to maintain cardiac output. The process that describes the electrical stimulation that ultimately leads to CM contraction and force generation is termed cardiac excitation–contraction coupling.[1] During this process, an electrical impulse (action potential) stimulates the release of intracellular Ca^{2+} from the sarcoplasmic reticulum. Ca^{2+} then binds to the troponin complex triggering the shifting of tropomyosin which exposes binding sites on actin filaments allowing myosin heads to bind actin and generate force (**Figure 1**). Though the mechanisms that regulate the amount of contractile force CMs generate are complex, at the basic molecular level, changes to contractile force are governed by the interaction between the sarcomeric proteins actin and myosin. As more myosin heads bind to actin filaments, force production increases. Conversely, a reduction in myosin heads bound to actin filaments will result in less than optimal contractile force. While this is true for healthy hearts, cardiac dysfunction resulting from heart attacks, arrhythmias, cardiomyopathies, or cardiotoxicity can lessen the ability of CMs to modulate contractile force in response to environmental changes.[2–6]

In addition to contracting and creating force to move blood, CMs must be able to relax and maintain an appropriate resting tension that allows for proper ventricular filling and cardiac function.[7] In CMs, resting tension is mainly governed by the elastic sarcomeric protein titin (Figure 1), but is also impacted by cytoskeletal proteins including microtubules, and extracellular proteins such as collagen.[8–12] When CMs are stretched beyond their resting tension, they develop a passive force that impacts cellular stiffness. Reports have shown that titin is responsible for up to 90% of the passive forces CMs experience.[8,13–15] Thus, this sarcomeric protein is a primary determinant of CM stiffness. Titin's impact on cellular stiffness is mainly dictated by the expression ratio of the small (N2B) to large (N2BA) isoforms of the protein (Figure 1). CMs that experience high passive stiffness correlate with a higher expression ratio of the smaller stiffer N2B isoform, whereas more compliant CMs have a higher ratio of the larger more compliant N2BA isoform.[16–18] While the impact of heart attacks and cardiomyopathies that result in heart failure on cardiac stiffness and contractile force has been extensively studied,[19–22] it remains unclear how cardiotoxic drugs alter these properties.

Dr. C. A. Blair, Prof. B. L. Pruitt
Department of Mechanical Engineering
University of California Santa Barbara
Santa Barbara, CA 93109, USA
E-mail: blp@ucsb.edu
Dr. C. A. Blair, Prof. B. L. Pruitt
Biomolecular Science and Engineering Program
University of California Santa Barbara
Santa Barbara, CA 93109, USA
Prof. B. L. Pruitt
Department of Molecular, Cellular and Developmental Biology
University of California Santa Barbara
Santa Barbara, CA 93109, USA
The ORCID identification number(s) for the author(s) of this article can be found under https://doi.org/10.1002/adhm.201901656.

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In this review, we first highlight the importance of human-induced pluripotent stem cell-derived cardiomyocytes (hiPSC-CMs) for cardiotoxicity studies and define cardiotoxic processes and agents. Then, we provide a brief overview of techniques that can be used to quantify changes in the forces CMs actively generate and passively experience in response to cardiotoxic drugs. We will focus on assays that have utilized live cell models because the advent of hiPSC-CMs promises a source of CMs that can be matched to the genetic background of patients and can display similar physiology to adult human CMs.[23–25] We conclude by discussing the application of these tools in understanding how cardiotoxic drugs impact the mechanobiology of CMs, or how CMs sense and respond to mechanical load at the cellular level.

2. hiPSC-CMs as Models to Assess Cardiotoxicity

One of the challenges when conducting drug screening assays for cardiotoxicity is the availability of cellular models that can recapitulate the physiology of adult human CMs in vitro. While primary human CMs can be isolated from explanted hearts for these assays, the propagation of these cells in vitro has been difficult. The use of cell lines from animal models that overexpress human cardiac ion channels have also been applied to cardiotoxicity studies,[26] but these models are often inaccurate at predicting human cardiac drug responses as the physiology of these cells differ from humans. HiPSC-CMs overcome many of the challenges with current models as they closely recapitulate the physiology of adult human CMs, can be readily differentiated and can be maintained for months in culture.[27–29] Moreover, with growing recognition of the long-term cardiotoxic effects of some drugs on subcellular structure and function, there is increasing interest in using hiPSC-CMs for cardiotoxicity studies. hiPSC-CMs not only address many challenges of physiology mismatch with animal models and

Cheavar A. Blair is currently a postdoctoral fellow at the University of California Santa Barbara. He graduated from Southern Illinois University at Carbondale with a B.S. in biology and an M.S. in plant biology. He received his Ph.D. degree in physiology from the University of Kentucky where he studied the mechanical function of human cardiac tissue. His current research interests focus on understanding how cardiotoxic agents impact the mechanobiological function of cardiomyocytes.

Beth L. Pruitt graduated from the Massachusetts Institute of Technology with an S.B. in mechanical engineering. She received an M.S. in manufacturing systems engineering from Stanford University. She earned her Ph.D. in mechanical engineering at Stanford University where she specialized in microsystems and small-scale metrologies. She was a postdoctoral researcher at the Swiss Federal Institute of Technology Lausanne (EPFL) where she worked on polymer devices. She is an elected Senior Member of IEEE, and a Fellow of the ASME and AIMBE.

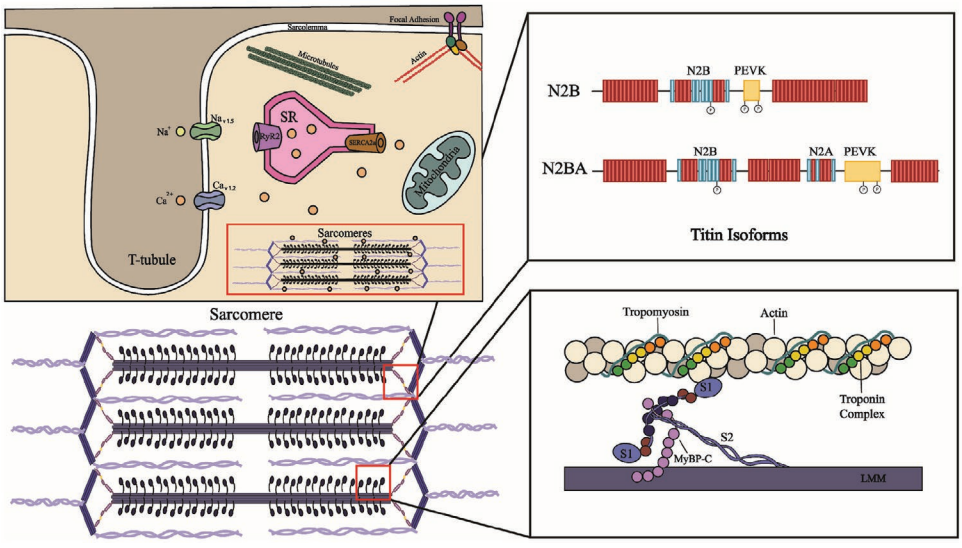

Figure 1. Cellular structures that impact passive stiffness and force generation in CMs. Representative CM with cellular organelles, ions, and protein structures (top left). The two isoforms of titin found in CMs (top right). Proteins within the sarcomere that are involved in force generation (bottom right).

primary cell viability, they also offer the potential to study electrophysiological effects and changes in cardiomyocyte contractile function and mechanical in response to cardiotoxic drugs.

3. Cardiotoxic Agents and CM Function

The cardiotoxic side effects of drug therapies were first described in the 1940s when it was noted that treatment with digitalis and local anesthetics adversely impacted cardiac function.[30] Today it is widely known that treatment with chemotherapies such as anthracyclines (e.g., doxorubicin), and other drugs including antiarrhythmic (e.g., quinidine), antipsychotic (e.g., chlorpromazine), and antihistamines (e.g., astemizole) can cause cardiotoxic side effects.[31–34] Among these agents, the most concerning are chemotherapies, as there is increasing recognition that traditional (e.g., doxorubicin and paclitaxel) and novel (e.g., trastuzumab, sunitinib, pembrolizumab) cancer drugs are leading to cardiovascular toxicity and heart failure.[35–39] While the exact mechanism of cancer drug-induced cardiotoxicity is not well understood, it is clear that chemotherapies can adversely impact several aspects of cardiac function, including electrophysiology, mitochondrial function, contractility, and viability (**Table 1**).[40–45] Changes to these structural and functional components can, in turn, alter the intrinsic ability of CMs to contract and generate active force independent of external load or applied stretch.

Proarrhythmic drugs are also of concern, as these drugs can lead to the elongation of the QT interval, which in rare cases can cause a potentially fatal ventricular arrhythmia known as Torsade de Pointes (TdP).[46] Drug-induced TdP occurs when a patient is treated with proarrhythmic drugs that block the potassium channel encoded by the human *ether-a-go-go* related gene (hERG), resulting in delayed ventricular repolarization.[47] In the late 1980s to early 2000s, several drugs (e.g., lidoflazine, terodiline, cisapride, terfenadine, etc.) were withdrawn from the market because of proarrhythmic concerns, as it was shown that they can prolong the QT interval and induce TdP in patients.[46–49] As a result, regulatory bodies including the FDA, and the pharmaceutical industry have worked together to create initiatives to outline methodological approaches to evaluate and identify drugs with QT liability during preclinical and clinical studies.[46] For example, the International Council on Harmonization of Technical Requirements for Registration of Pharmaceuticals for Human Use (ICH) has created a guideline (ICH S7B) for nonclinical evaluation of drugs that block hERG or cause QT prolongation using in vitro and in vivo models. Since the implementation of the ICH S7B guidelines, there have not been any drugs approved that block hERG or prolong ventricular repolarization in patients.[31] However, as a result of the high sensitivity and low specificity of the assays used to determine the impact of drugs on hERG channels and QT prolongation, several drugs that have little risk of causing TdP are deprioritized or have been excluded from further development.[31] Another initiative that has been created is the comprehensive in vitro proarrhythmia assay (CiPA), which was established to develop a new in vitro paradigm for assessing the proarrhythmic risk of new drugs. The vision of CiPA is that drugs that are unlikely to cause TdP and little or no QT prolongation will be labeled as benign instead of being disqualified from the drug development process.[31] To accomplish this, CiPA aims to define the effects of drugs on multiple human cardiac currents (not only hERG), use in silico reconstruction of the ion channel effects to determine net effects on electrophysiology and verify the effects of proarrhythmic drugs using hiPSC-CMs.[31] To date, the efforts of CiPA and several initiatives have resulted in a proarrhythmic clinical risk categorization system that ranks (high, intermediate, or low) proarrhythmic drugs according to their potential of causing TdP (Table 1).

The implementation of guidelines for the preclinical assessment of drug effects on the action potential of CMs using in vitro assays has paved the way for patch-clamping microelectrode arrays (MEAs), and other electrophysiological assays to serve as the primary standard for preclinical in vitro cardiotoxicity screening. It is now routine when conducting cardiotoxicity studies to report data gathered from electrophysiological techniques (e.g., patch-clamping, MEAs, voltage dyes, etc.). Though these techniques are imperative for understanding the cardiotoxic effects of a drug, they are outside the scope of this review and have been extensively reviewed by others. For further reading, we refer the reader to these reviews.[34,47,50]

The impact of proarrhythmic and cancer drugs on the electrophysiological function of CMs is evident, it is less clear how cardiotoxic agents impact the mechanical function, structural components, and extracellular environment around CMs that can alter passive forces. These studies are needed because cardiovascular disease related to oncology therapies is of increasing concern for emerging cancer therapies.[51] Such structure–function studies will provide a more complete picture of how cancer drugs and other toxic compounds alter the active and passive forces of CMs and enable therapeutic interventions to detect and mitigate the secondary effects of cardiac dysfunction and heart failure.

4. Assays Used in Cardiotoxicity Studies to Measure Active Force

The active force CMs generate is driven by the interaction between the sarcomeric proteins actin and myosin. These proteins are able to interact and produce force when Ca^{2+} ions bind the troponin complex which through a series of events opens binding sites on actin filaments that allow myosin heads to attach and pull actin filaments inward to contract the cell and generate force (Figure 1). Several assays have been reported to measure changes in active forces in response to cardiotoxic compounds in vitro at the cellular and tissue levels. The functional readout of contractile "force" is commonly reported (**Table 2**). Importantly, force is always derived from displacement measurements coupled with mechanical models and underlying assumptions about material properties. For example, traction force microscopy (TFM) tracks the displacement of fiducials (beads) in a substrate using high-speed video to determine changes in the amplitude of a contractile field exerted by a cell. These displacements are then mapped to forces by assumptions about mechanical properties and boundary conditions.[93–97] From these forces and deformations, one can estimate the work

Table 1. Example comprehensive in vitro proarrhythmia (CiPA) and cancer drugs that have been identified as cardiotoxic.

CiPA drugs	Mechanism of action in cardiomyocytes	Effects on electrophysiology	Effects on contractility	Effects on viability/O^2 consumption
Dofetilide (high[a])	Block hERG encoded I_{Kr} (delayed rectifier potassium) channels	Prolong QT interval and Increase APD[40,52]	Enhance contractility in rat ventricular cells[53]	No reports
Quinidine (High[a])	Blocks fast I_{na}, slow Ca^{2+}, and hERG encoded I_{Kr} channels[54]	Increase APD in patients[55]	Reduce beating amplitudes in hiPSC-CMs monolayer[56]	Reduces oxygen consumption in rat heart slices[57] Cardiotoxic to hiPSC-CM[58]
Sotalol (high[a])	Nonselective beta-adrenergic blocker[59] Blocks hERG encoded I_{Kr} channels[60]	Increase APD in patients[61]	Lower contractility in dog hearts[62]	No reports
Cisapride (intermediate[a])	Blocks hERG encoded I_{Kr} channels[63]	Increase QT interval and ADP[b] in patients[64]	Do not alter contractility in human primary cells[65]	Reduces cell viability[66]
Terfenadine (intermediate[a])	Blocks hERG encoded I_{Kr}[67] and I_{Na}[68] channels	Prolongs QT interval in hiPSC-CM monolayer[69]	No effect on contractility in hiPSC-CM monolayer[69]	Reduce cell viability in hiPSC-CM monolayer[70]
Chlorpromazine (intermediate[a])	Blocks hERG encoded I_{Kr}[71]	Prolongs QT interval in guinea pig ventricular myocytes[72]	Reduced contractility in human myocytes[65]	No reports
Nifedipine (low[a])	L-type Ca^{2+} channel blocker[73]	Decrease APD and Ca^{2+} transient amplitude in hiPSC-CM monolayer[74,75]	Lowers relative force in hiPSC-CM monolayer[76]	Does not alter O^2 consumption in rodent hearts[77] Protect chick hearts from apoptosis[73]
Verapamil (low[a])	Ca^{2+} channel blocker[78]	Low doses prolong APD High doses shorten action potential duration[78]	Reduce contractility and sarcomere dynamics in hiPSC-CM monolayer[79] Depress myofibril formation[80]	Increase myocardial oxygen supply in rodents[81]
Cancer drugs	**Mechanism of action in cardiomyocytes**	**Effects on electrophysiology**	**Effects on contractility/ myofibrillar structure**	**Effects on viability/O^2 consumption**
Trastuzumab	Monoclonal antibody that targets HER2 receptor[82]	May induce arrhythmias in patients[83]	Decrease left ventricular ejection fraction in patients[84]	Reduce cell viability[82]
Sunitinib	Tyrosine kinase inhibitor[85]	Prolongs QT interval in humans[86]	Declines contractile force in engineered heart tissues made with rat heart cells[87]	Reduces mitochondrial function, and is cardiotoxic[85]
Sorafenib	Tyrosine kinase inhibitor	Reduce Ca^{2+} concentration and re-uptake in the sarcoplasmic reticulum in human cardiac tissue[88]	Decrease force development in human cardiac samples[88]	Cardiotoxic[88] Reduce oxygen consumption in rat hearts[89]
Imatinib	Tyrosine kinase inhibitor	No effect on APD in hiPSC-CM monolayer[38]	Declines contractile force in engineered heart tissues made with rat heart cells[87]	Does not alter cell viability in hiPSC-CMs[38]
Paclitaxel	Stabilizes tubulin polymerization	Increase spontaneous Ca^{2+} oscillations in mice[90]	Reduced mechanical output, depressed myofibril formation[44,45]	No effect on cell viability in rodents[90]
Doxorubicin	Prevents DNA re-ligation and double-stranded brake repair	Impair Ca^{2+} handling,[91] increase intracellular Ca^2 in hiPSC-CM monolayer[92]	Reduce contractility, degrades myofibrils[35,43]	Impair metabolic function; reduces O^2 consumption in hiPSC-CM monolayer[91]

[a]CiPA TdP risk ranking; [b]ADP, action potential duration.

or strain energy in a contractile cycle. Several other assays focus on ensemble cell motions or edge displacements and estimate a more limited set of biophysical parameters like rates and connectivity. Below, we review in order of prevalence, how several such mechanobiology assays and parameters have or could be used to answer questions about the impact of cardiotoxic agents on active force.

4.1. Video Microscopy

High-speed (typically >40 Hz) video capture of the contraction and relaxation cycles is a commonly used technique to assess changes in the displacement of CMs in response to cardiotoxic agents (**Figure 2**). These measurements are frequently performed in drug screening and development assays

Table 2. Mechanobiological techniques currently used to assess changes to CM structure and function using live-cell models.

Technique	Mechanical readouts	Electrophysiological readouts	Structural readouts	Applicable platforms	Strengths	Limitations	Refs.
Video microscopy	Beating frequency Time to peak contraction Time from peak to 50% and 90% relaxation	Action potential duration[a),b)] Action potential amplitude[a),b)] Mean diastolic potential[a)] Field potential[a)] Spike slope[a)] Spike amplitude[a)] Membrane potential[a)]	Sarcomere length[c)] Sarcomere shortening percentage[c)] Relaxed/contracted sarcomere length[c)]	Single-cell 2D monolayer 2D organized sheet 3D tissue constructs	Noninvasive High-throughput Low level of entry for new users	Cannot quantify force Analysis of displacement can vary based on analysis software	[107,108,119, 156–159]
Traction force microscopy (TFM)	Beating frequency Beat displacement Relative force Contraction/relaxation times Velocity of contraction/relaxation Power Force–frequency	Action potential duration[b)] Action potential amplitude[b)] Resting membrane potential[b)]	Sarcomere length[d)] Sarcomere shortening percentage[d)] Sarcomere registry[d)] Myofibril alignment[d)] T-tubule formation[d)]	Single-cell 2D monolayer 2D organized tissue 3D tissue constructs	Can tune substrate stiffness Quantification of relative force Noninvasive	Computationally intensive Low throughput	[121–124,132,160]
Micropost arrays/micropost for engineered heart tissues (EHT)	Beating frequency Beat displacement Relative force Contraction/relaxation times Velocity of contraction/relaxation Power Force-frequency	Action potential duration[b)] Action potential amplitude[b)] Resting membrane potential[b)] Conduction velocity (EHT) Upstroke velocity (EHT)	Sarcomere length[e)] Myofibril alignment[e)] Sarcomere banding[e)] T-tubule formation[e)] Mitochondrial distribution[e)] Location of gap junctions[e)]	Single cells Monolayers 3D tissue constructs	Simple force calculations No reference image required EHTs closely recapitulate the morphology, structure, and electrophysiology of human tissue	Difficult to replicate physiological stiffness Discrete rather than continuous adhesion interface (array) Large number of cells per tissue (EHT) Low throughput	[133,139,140,144, 145,161–163]
Atomic force microscopy (AFM)	Beating frequency Vertical force Relative force (when combined with TFM) Cellular stiffness	Action potential duration[b)] Action potential amplitude[b)]	No published reports	Single-cell 3D tissue constructs	Quantification of force and cellular stiffness	Requires technical expertise Low throughput	[154,155,164–166]

[a)]Electrophysiological readouts reported when combining video microscopy with patch clamping or microelectrode arrays (MEAs); [b)]Electrophysiological readouts reported when combining video microscopy with fluorescent dyes or genetically encoded voltage sensors that binds ions including Ca^{2+} (e.g., GCaMP, Fluo-4, and FluoVolt); [c)]Structural readouts of sarcomere dynamics reported using the SarcTrack analysis software developed by Toepfer et al. [119]; [d)]Structural readouts using single-cell CMs, as reported by Ribeiro et al.[120,132]; [e)]Structural readouts from EHT systems using electron microscopy or fluorescent markers and dyes.

in conjunction with electrophysiological measurements.[32,98] Although the recording of videos for these assays is straightforward, data analysis is more complex. In contrast to isolated primary CMs from animal and human heart tissue, unstructured hiPSC-CMs lack a defined cellular membrane, sarcomere organization, and direction of contraction.[99,100] Macroscale motions can be mapped using particle tracking algorithms,[101–103] and while motion maps can be extracted from such data, many material and boundary assumptions must be made to transform to force estimates. Since, monolayers of hiPSC-CMs or unstructured single hiPSC-CM lack well-defined morphological features, these motion analysis methods have difficulty accurately tracking and quantifying changes in displacement in these models. With the adoption of hiPSC-CMs as models for drug development and screening,[104–106] newer analysis methods seek to account for the lack of organized sarcomere, nonuniformity of contraction, and lack of pronounced membrane edges.[99,100,107–109] Such algorithms track displacement in hiPSC-CMs either by monitoring changes in pixel intensity or applying particle image velocimetry methods to generate vector maps that track displacement in all directions regardless of sarcomere organization or detectable edges.[101,109,110] This is fundamentally different from analysis tools that relied on raster-line scanning of periodic features and edges (as found in primary cells) to detect changes in sarcomere motion or the displacement of the edges of CMs.[111–113]

Several labs have deployed advanced video microscopy analyses of drug-induced changes in contractile function of hiPSC-CMs, from single cells to 3D tissue constructs.[91,98,108,114–116] Common mechanical readouts include changes in peak contraction/relaxation amplitudes, contraction/relaxation velocities, and beat rate (Table 2). For example, Sala et al. deployed their

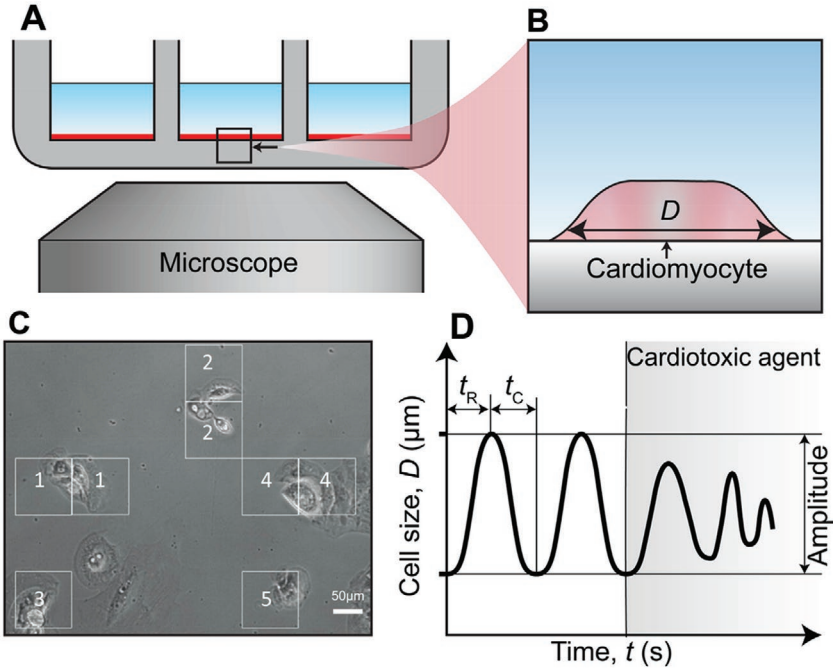

Figure 2. Representative workflow of video microscopy technique used to gather functional and structural data from CMs. A) Microscope with a camera used to capture high speed videos of beating CMs. B) Isolated CM illustrating the change in displacement over time. C) Representative video analysis software used to quantify functional changes in CM displacement over time. Reproduced with permission.[107] Copyright 2015, Elsevier. D) Representative functional readout used to quantify changes in displacement over time.

"MUSCLEMOTION" analysis software to study the cardiotoxic agent Nifedipine (a Ca^{2+} channel blocker used to treat hypertension) and learn that it reduces contraction amplitudes in single-cell hiPSC-CM, hiPSC-CM monolayers, and 3D tissue constructs.[108] Similarly, Maddah et al. used their "Pulse" video analysis tool to observe a reduction in the beat rate of hiPSC-CMs monolayers exposed to the cardiotoxic agents verapamil (Ca^{2+} blocker) and cisapride (a drug used to treat gastric-emptying disorders).[107]

Live cell models can also be coupled with Ca^{2+} dyes,[117] genetically encoded voltage sensors,[118] and fluorescently labeled organelles (e.g., mitochondria) or structural proteins (e.g., sarcomeres). Video analysis tools can then correlate structural parameters with Ca^{2+} handling, electrophysiological function, structural damage or disorganization, and mechanical parameters (see Table 2). Changes to electrophysiological function, Ca^{2+} handling, or cellular damage can greatly impact the amount of active force CMs can generate. "SarcTrack" is one such video analysis tool that tracks the displacement of fluorescently labeled sarcomeric proteins to reveal changes to sarcomere length and shortening.[119] Not surprisingly, sarcomere length changes have been correlated with contractile force.[120] Toepfer et al. used of SarcTrack to detect changes to sarcomere function in response to drug treatment using a small molecule that alters myosin function.[119] Monitoring such changes at the level of sarcomere structure and function will enable new insights into mechanisms of action of cardiotoxic compounds. With continued improvements in imaging tools and analysis software, video microscopy analysis will continue to be a cornerstone of drug discovery and screening assays. When combined with quantitative assays of hiPSC-CM contractile work output, such assays offer the potential for semiautomated pipelines to assess change to contractile function under drug treatments.

In the following sections, we focus on biomechanical assays that when combined with molecular biology manipulations and video microscopy, enable insight into the mechanobiology of cardiotoxic responses. By mechanobiology, we mean the changes in biophysical phenotype and the role of mechanical load in cell signaling and in exacerbating cell responses to cardiotoxic compounds.

4.2. Traction Force Microscopy

TFM is a technique that is used to quantitatively determine the amount of force CMs exert against a substrate by monitoring the displacements of fiducials embedded in the substrate (**Figure 3**). Video microscopy alone provides data on changes in contractile displacements and rates (Table 2) by measuring the displacement of CMs over time. By capturing an additional video channel of fluorescent bead motions in a deformable substrate under a cell, TFM can be used to quantify active force, power, and strain energy that CMs exert on a substrate. We refer interested readers to several excellent reviews on the instrumentation and device preparation required, image-processing algorithms, and subsequent postprocessing analysis principles for TFM.[121–124]

TFM can be used to determine the active forces generated by single cells and 2D sheets of CMs, and with appropriate model construction and confocal imaging, within 3D tissue constructs.[125–129] Because of the postprocessing computation required, TFM has not been widely adopted for drug discovery and screening. However, unlike high-throughput video microscopy assays performed on plastic or glass, TFM offers advantages for research into mechanobiology mechanisms because the substrate stiffness can be matched to physiological stiffness in disease and development. Substrate stiffness is a key factor in functional measurements on CMs, as the stiffness of the substrate can significantly impact the amount of active forces hiPSC-CMs generate.[120,130,131] Thus, performing TFM using substrate stiffnesses similar to the physiological environment can be advantageous for examining the impact of drugs on CM mechanical function. TFM is also readily combined with protein patterning techniques to constrain hiPSC-CM size and shape,[120] such cell patterning enhances the subcellular structure, maturity, and biophysical function.

TFM has been deployed in drug screening assays to demonstrate the impact of several compounds on CM function.[100,108] For example, Kijlstra et al. used TFM and reported a reduction in single-cell hiPSC-CM active force production in response to verapamil, and dofetilide (antiarrhythmic drug), two known

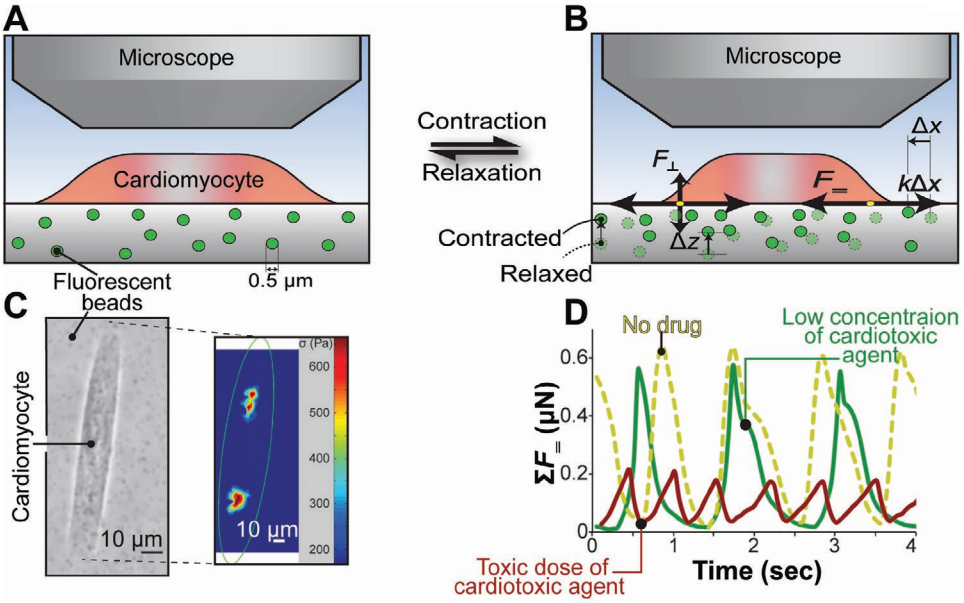

Figure 3. Traction force microscopy (TFM) of A) relaxed and B) contracted CM on hydrogel substrate with fiducials. C) Representative bright field and traction force map of single-cell CM. D) Representative traces of the summed traction forces CMs generate in response to drug treatment. (C,D) Reproduced with permission.[132] Copyright 2017, Wolters Kluwer Health, Inc.

cardiotoxic agents.[100] We have also used TFM to show the application of the technique in assessing the effects of contractile agonists and stimulants on force generation in single-cell hiPSC-CMs.[132] With the advantages TFM offers over traditional video microscopy assays in terms of quantitative measurements and substrate stiffness manipulation TFM holds the potential to examine underlying mechanisms and the impact of cardiotoxic agents on the active forces CMs produce.

4.3. Microposts for Single-Cell and Engineered Heart Tissue Force Measurements

Micropost platforms use vertical, deformable beams to estimate the force of attached CMs (**Figure 4**). This mechanobiological assay converts micropost motion (imaged at the top of the post) to force using a simpler mechanical model for beam bending and assumptions about the material properties of the microposts. Pairs of posts have been used with 3D cardiac tissue constructs encircling the posts; while arrays of microposts have been used to study monolayers and single CMs attached to the top of the posts (Figure 4). Micropost arrays have been used by several labs to measure the contractile forces generated by single-cell CMs.[133–139] This technique is very similar to single-cell TFM assays in that the traction forces generated by CMs create displacement in the substrate. These displacements are used to calculate the amount of active force from the known spring constant of the beams.[133] Micropost fabrication does require specialized tools or access to a lithographically defined mold. Once manufactured, however, microposts are relatively shelf-stable compared to TFM hydrogel substrates that change stiffness appreciably in about a week.[140] Another advantage is that post displacement is directly related to the force CMs apply to that individual post, making force calculations straightforward and less computationally intensive.[121] However, microposts also present challenges. Matching the stiffness of microposts to physiological conditions is not straightforward because they have an effective out of plane stiffness that is an order of magnitude stiffer than in-plane stiffness. They also present a discontinuous biointerface where cells can sag or engulf the microposts depending on the protein functionalization strategy.

Microposts are also used to measure changes in the active forces generated by 3D cardiac tissue-like constructs commonly referred to as engineered heart tissues (EHTs). Unlike the micropost arrays used in single-cell measurements (Figure 4), microposts used in EHTs studies use two posts that serve as a scaffold to hold and measure the displacement of the EHT (Figure 4). The EHT is typically cast from a cell–matrix suspension on the substrate and forms a continuous tissue around the microposts. The EHT creates a preload with tissue compaction and slides to the top of the posts, thus, the posts typically include features at the top to keep the EHT from slipping off the top of the posts during contraction. The use of EHTs in drug screening and developmental assays is of increasing interest with commercial systems now coming to market.[141,142] The hiPSC-CMs in EHTs "trained" with intervals of electrical pacing more closely resemble CMs in an intact heart compared to CMs in 2D monolayer and unstructured single-cell models.[143] In the past decade, there have been several studies that have reported changes in the contractile forces produced by EHTs in response to cardiotoxic agents identified by the CiPA initiative as well as cancer drugs using micropost displacements (Table 1).[144–149] For example, Hansen et al. showed that the cardiotoxic agents quinidine, chromanol, erythromycin, and doxorubicin all negatively impacted CM function using EHTs.[149] With continued improvements in EHT micropost, their use is likely to grow in drug screening assays.

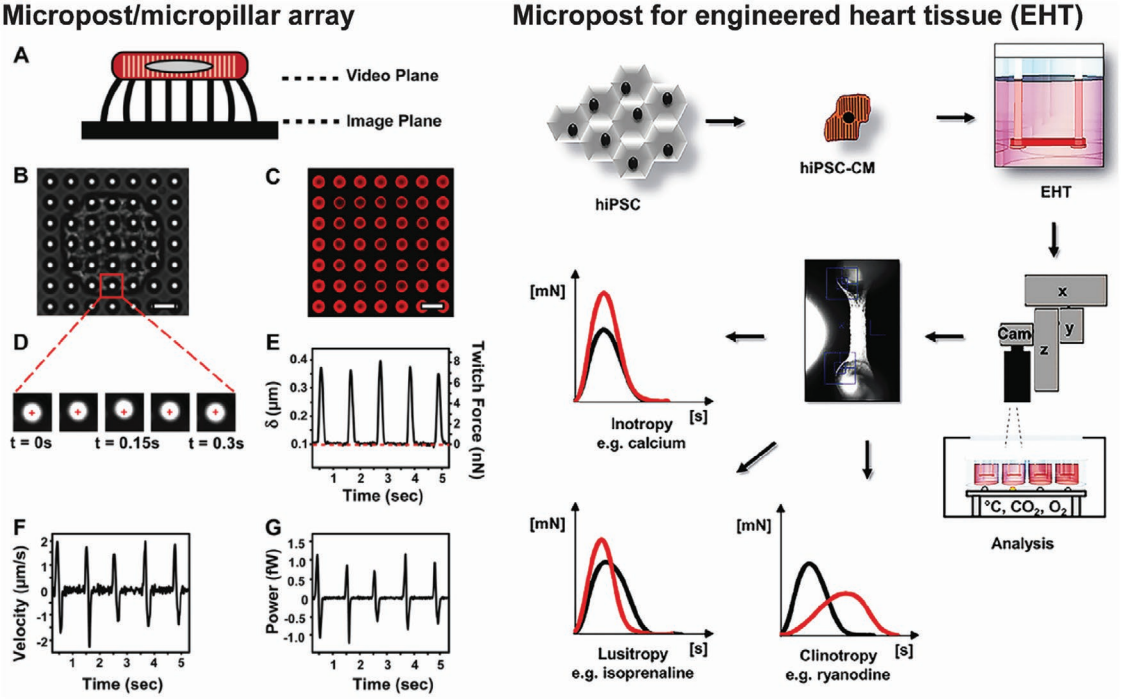

Figure 4. Micropost platforms. (Left) Micropost/micropillar array for single-cell mechanical measurements and analysis workflow. Reproduced with permission.[104] Copyright 2014, The Society of Mechanical Engineers. A) Depiction of videos taken during micropost experiment using high-speed camera, video plane is recorded using phase contrast of the tip of micropost and image plane of the base of micropost is recorded in a fluorescent channel. B) Phase contract video at the tip of microposts for tracking posts displacement. C) Fluorescent image of the base of micropost to establish a reference position. D) Custom software used to locate and track the centroid of each micropost to determine micropost deflections over time (plus sign), as well as track the location of the post's centroid in the video plane. Differences in two centroids are then used to determine E) CM twitch force, F) the velocity of post deflection, and G) calculate power. (Right) Example workflow of micropost used in engineered heart tissue (EHT) measurements to determine the impact of drugs on mechanical function. Reproduced with permission.[144] Copyright 2016, Elsevier.

4.4. Atomic Force Microscopy (AFM)

Atomic force microscopy is a quantitative assay that was developed to probe nanoscale features of solid materials but is increasingly being applied to biology. AFM measures the interaction forces of a cantilever tip with a surface from the deflection of a cantilever with a known spring constant (**Figure 5**). The cantilever can be brought into contact with a cell to measure cell biomechanical properties. However, beating hiPSC-CMs also exert force on the free end of the cantilever, the active vertical force produced by the CM causes the displacement of the cantilever, and the lateral forces of contraction can be measured as well as changes in cell stiffness.[150] Several studies have used AFM to study the mechanical function of CMs.[151–155] However, few have used AFM to examine the impact of cardiotoxic agents on the active forces CMs produce. For example, AFM was used to study cardiotoxic reductions in CM force induced by the chemotherapeutic agent doxorubicin.[154,155]

Although AFM has been used to study CMs and cells in general, it is not a widely adopted technique for assessing changes to the active forces these cells produce. AFM is not high-throughput and force measurements on CMs are challenging and require specialized equipment and technical expertise. The beating nature of CMs can also create fluidic disturbances that can impair accurate force measurements.[153] Traditional AFM can only measure one cell at a time, and it is not trivial to reposition the cantilever accurately for measurements on multiple cells. Thus, while AFM has the potential for probing mechanisms linked to changes in cell mechanical properties, it has limited application for high-throughput drug development or screening assays.

5. Measuring Changes to CM Stiffness in Response to Cardiotoxic Agents

CMs have an inherent stiffness that is mainly governed by the expression ratios of the N2BA (more compliant) and N2B (stiffer) isoforms of the elastic sarcomeric protein titin (Figure 1).[167] In healthy adult hearts, the expression ratios of N2BA:N2B in CMs are 30:70 to 40:60 which provides the relatively high tensile stiffness needed for proper cardiac function.[168] Studies have shown that the expression ratio of these two isoforms shifts during developmental and diseased states, with the titin-based passive tension getting higher/stiffer throughout development (switch to more N2B) and more compliant (switch to more N2BA) with disease.[19,169–171] In addition to isoform switching, mutations in the titin protein that leads to truncation or altered phosphorylation can also alter CM stiffness and lead to cardiac dysfunction.[172–175] Changes to the stiffness individual CMs to a lesser degree can also be impacted by microtubules, as these cytoskeletal proteins have been shown to

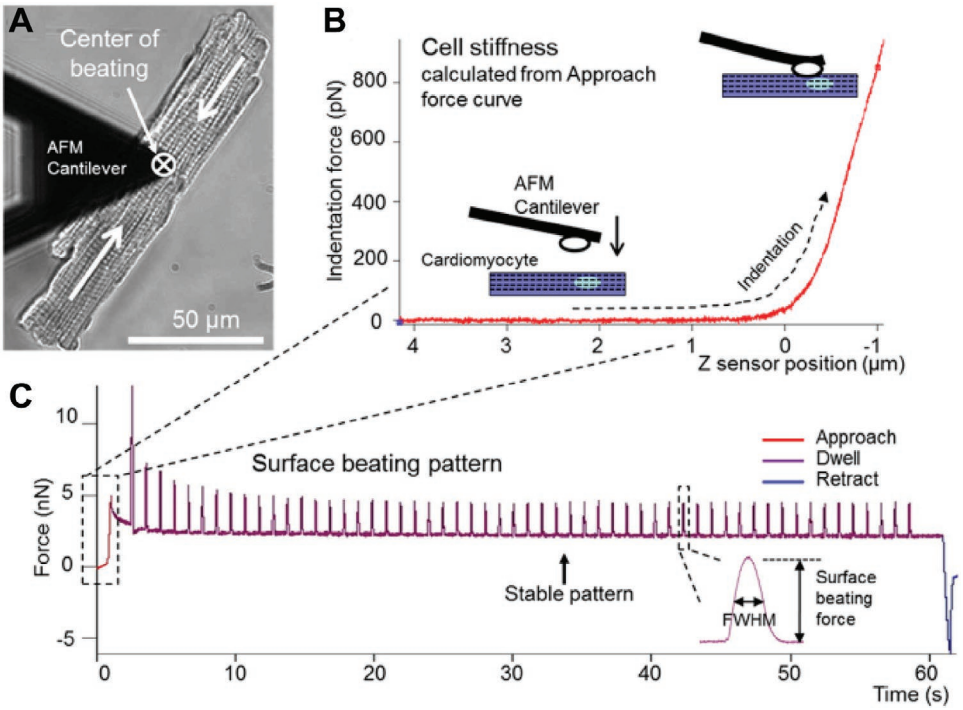

Figure 5. AFM measurement and data analysis. A) AFM cantilever aligned over the center of CM. B) Representative AFM data of an indentation curve used to determine CM stiffness. C) Representative data used to determine surface beating force. Reproduced with permission.[155] Copyright 2016, American Chemical Society.

alter the passive stiffness of CMs especially in response to post-translational modifications during disease states.[12,176]

Several techniques have been used to measure the stiffness of live individual CMs in vitro including AFM, carbon fibers, and microfluidics-based and traditional micropipette aspiration assays.[153,177–181] Of these methods, AFM is the most commonly used to assess stiffness changes in CMs. Similar to the active force measurements mentioned earlier, stiffness measurements using AFM are performed with a cantilever. To measure CM stiffness using AFM, the cantilever is used to create a precise indentation in the CM membrane that creates a force that is tracked along a force versus indentation curve. The data from this curve are then fitted to estimate a bulk elastic modulus for the cell (this procedure typically uses a Hertz model, though we note that cells are neither elastic nor homogeneous). Through a similar indentation method, the carbon fiber technique has also been used to determine the stiffness of CMs. However, instead of using a cantilever as in AFM, a small microsphere attached to the carbon fiber is used to create the indentation.[182] Micropipette aspiration is the oldest and simplest of these techniques. The technique is conducted by placing the tip of a pulled glass pipette on an individual cell and, by controlling applied suction, deforming the cell as it is pulled into the micropipette. The elastic properties of the cell are then inferred by Laplace's law and the changes in cell geometry. Micropipette aspiration was first used by Brady et al. in the 1970s to measure the mechanical properties of living individually isolated rat CMs using a single barrel pipette.[181] The technique has since evolved from using a single barrel pipettes to double-barrel pipettes (offers better cell attachment and wider scope of mechanical measurements) and in some applications, to semiautomated microfluidics with controllers to increase the precision and throughput.[178,183–185]

Of these, only AFM has been reported for assessing the impact of cardiotoxic agents on CM stiffness. For example, Yue et al. used the technique to show that the cancer drug doxorubicin reduced stiffness of isolated mouse primary CMs.[155] It was not reported in the study whether the reduction in stiffness was due changes to titin, microtubules or other cellular structures. However, patient data from breast cancer patients shows that cancer drugs can cause frameshift mutation in titin, reducing cardiac compliance and ultimately leading to cardiomyopathy.[186] More studies are needed to understand the cardiotoxic effects of chemotherapeutic and other cardiotoxic agents on CM stiffness to understand the mechanisms by which these compounds induce these changes.

6. Mechanotransduction and Cardiotoxicity

CMs must be able to sense mechanical loads in order to respond to their environment (changes in hemodynamics, ECM content, etc.). The mechanobiology processes by which these cells sense and transmit mechanical signals to initiate a biomechanical response are referred to as mechanosensing and mechanotransduction respectively.[187] There have been several proteins and complexes identified as mechanical sensors, signal transducers, and structural transmission conduits of mechanical loads in CMs, including integrins, the dystrophin–dystroglycan complex, the sarcomere, talin, and other elements of the

cytoskeleton.[188–190] In CMs, mechanotransduction of mechanical loads are essential to cellular function, development, and maladaptive responses, as the transmission of these signals throughout the cell can significantly alter cellular structure and function.[188] For example, Jian et al. have shown that mechanical load can significantly impact the action potential duration, calcium transients, and contractility of single CMs.[191]

The impact of cardiotoxic agents on the ability of CMs to sense and transmit stress and strain is not fully understood. However, reports from studies in rabbits and humans have demonstrated altered expression of integrins and titin genes, respectively, in response to cancer drug treatment.[186,192] In both cases, rabbits and humans developed heart failure. While it is unclear if the changes in the gene expression of integrins and titin resulted in altered protein function leading to any causal effects, it is widely known that changes in the function of integrins and/or titin can impact contractility and the transmission of mechanical signals.[188,193] More research in this area is needed to determine the effects of cardiotoxic agents on CM's ability to sense and transmit mechanical signals.

To address these questions, mechanobiological techniques will play an invaluable role as these assays can provide insights into the mechanisms CMs use to sense and transmit mechanical loads. For example, Pandey et al. used nanopillars (nanoscale post arrays) to show that CMs can sense matrix rigidity through a combination of muscle and nonmuscle myosin activity.[194] Using the same technique, they also demonstrated that the stretching of the protein talin which can impact mechanotransduction of mechanical signals depends on matrix stiffness, myofibrillar, and nonmyofibrillar tension.[194] While these investigators did not apply any cardiotoxic agents to the CMs in their studies, their work provides experimental data that mechanobiological techniques like post arrays can be used to answer questions on how CMs sense and respond to their environment and cardiotoxic agents.

7. Future Outlook

The use of hiPSC-CMs for cardiotoxicity studies will continue to grow as these cells more closely recapitulate the physiology and function of human adult CMs than in vitro cell models from animals. HiPSC-CMs also offer researchers the opportunity to study the impact of cardiotoxic agents on CMs with mutations, an area of research that is not heavily explored. The importance of these studies was recently highlighted by Lostal et al.[195] who used rodent and nonhuman primate models to demonstrate that the splice variant of the giant protein titin that is expressed in the heart can lead to cardiotoxicity by altering the buffering capacity of the heart to calpain 3. In their study, the team revealed that mice were more likely to develop cardiotoxicity as a result of calpain 3 AAV gene transfer in comparison to nonhuman primates because the titin isoform rodents express is unable to bind and sequester similar amounts of calpain 3. Though these researchers did not examine a particular mutation, they highlighted the importance of understanding the interplay between cardiotoxic agents and protein variants that are the result of alternative splicing or mutations.

While electrophysiological assays (e.g., patch clamp, MEAs, fluorescent dyes, etc.) to identify arrhythmogenic effects of cardiotoxic agents remains a primary standard for preclinical cardiotoxicity screening, assessing concomitant changes in the mechanical phenotype of CMs during the screening process is also needed to unravel mechanisms of disease progression.[196] As discussed in this review, one of the main challenges for adopting the current mechanobiological techniques in a preclinical drug screening process is their low throughput. Though techniques such as TFM and AFM can be used to probe CMs for mechanistic discovery, as well as provide a quantitative assessment of changes to CM function, they still require significant technical expertise. However, with the continued development and automation of these techniques, as well as other methods such as the EHT/micropost systems, mechanobiological assays to assess changes in CM function will eventually make their way in the drug screening and development process. Future work is also needed to bridge single cell, tissue, and organism level data to rationalize the outputs across these different scales.

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Conflict of Interest

The authors declare no conflict of interest.

Keywords

cardiomyocytes, cardiotoxicity, heart failure, human induced pluripotent stem cells, mechanobiology, mechanotransduction

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