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Electrical and Mechanical Strategies to Enable Cardiac Repair and Regeneration

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Abstract

Inadequate replacement of lost ventricular myocardium from myocardial infarction leads to heart failure. Investigating the regenerative capacity of mammalian hearts represents an emerging direction for tissue engineering and cell-based therapy. Recent advances in stem cells hold promise to restore cardiac functions. However, embryonic or induced pluripotent stem cell-derived cardiomyocytes lack functional phenotypes of the native myocardium, and transplanted tissues are not fully integrated for synchronized electrical and mechanical coupling with the host. In this context, this review highlights the mechanical and electrical strategies to promote cardiomyocyte maturation and integration, and to assess the functional phenotypes of regenerating myocardium. Simultaneous micro-electrocardiogram and high-frequency ultrasound techniques will also be introduced to assess electrical and mechanical coupling for small animal models of heart regeneration.

Index Terms

Heart regeneration; electrical strategies; mechanical strategies; cardiomyocytes; maturation; cardiac repair

I. INTRODUCTION

The heart is one of the most dynamic but least regenerative organs in the body [1]. Myocardial infarction (MI) results in an irreversible loss of cardiomyocytes (CMs) [1, 2].

Injured human hearts repair through scarring, but the fibrotic tissue lacks the contractile phenotype, thus becoming a substrate for ventricular remodeling, arrhythmia, and heart failure [3–5]. The last decade has attested emerging tissue engineering strategies to unravel innate regenerative responses and to establish embryonic or induced pluripotent stem cells-derived CMs for cell-based therapy [1, 2]. The lessons taught from animal models such as zebrafish or neonatal mice [6–9] have provided fundamental understandings of the regenerative capacity of myocardium as a basis for cell transplantation strategies [10–12]. In parallel, the inception of engineered heart tissue (EHT) and *in-vitro* assessment of CM phenotypes have facilitated transplantation in animal models [10, 13–19]. Various methods for EHT have been developed, including the use of hydrogel technique, prefabricated matrices, decellularized cardiac tissues, and cell sheets [14, 20–26]. These advances have further paved the way for *in-vitro* heart regeneration studies, disease modeling and drug testing.

Despite the advances in stem cell-based transplantation, the translation of the results for pre-clinical studies and clinical trials remains a bottleneck. In 2012, John B. Gurdon of the U.K. and Shinya Yamanaka of Japan shared the Nobel Prize in Physiology and Medicine for their groundbreaking discoveries in reprogramming mature cells to become pluripotent. Their work has spawned a myriad of new studies in reprogramming adults cells to embryonic stem cells (ESCs) and inducing pluripotent stem cells (iPSCs)-derived human CMs [1, 16–19, 21, 27–29]. Tissue engineering approaches to provide micro-environmental cues have furthered enabled stem cell-based therapy [10–12, 30–32]. However, the immature phenotypes of ESC-CMs or iPSC-CMs can be a potential nidus for arrhythmogenicity and immunogenicity, hampering successful integration of transplanted CMs to the host myocardium [10, 20, 30, 32–37].

In the last decade, specialized electrical and mechanical bioreactors [35, 36, 38–51] have been developed to “train” the CMs towards mature phenotypes in animal models [35, 45, 46, 52, 53]. Integrating embedded nanostructures into the three-dimensional (3-D) scaffolds has further recapitulated the micro-environment in which electrical or mechanical stimulations promote sarcomere alignment and gap junction protein (*Cx43*) expression [32, 47, 54]. These electrical- and mechanical-based bioreactors have also been applied to induce ESC-CMs and iPSC-CMs towards adult phenotypes for drug discovery, toxicity screening, and cardiac disease modeling [37].

Successful animal models and clinical trials for cardiac repair cannot be accomplished without recovering functional phenotypes of myocardium after cell-based transplantation. In this context, electrical and mechanical strategies hold promise to provide approaches to validate mature CM phenotypes of regenerating myocardium [5, 6, 20, 21, 27, 30, 52, 55, 56]. Our research team has applied micro-electrocardiogram (μECG) and high-frequency ultrasound techniques to provide minimally-invasive and real-time monitoring of ventricular compliance and electrical repolarization [57–59]. Collectively, this paper has dual goals: (1) providing current electrical and mechanical stimulation approaches to address CM maturation critical for cell-based therapy, and (2) presenting methodologies to assess electrical and mechanical phenotypes important for cardiac functions.

II. ELECTRICAL AND MECHANICAL STIMULATIONS

A. Electrical Stimulation

The contractile properties of natural myocardium are intimately linked with the cellular and sarcomere orientation and elongation. Engineered CMs were observed to contract spontaneously with varying rates and regularity. Electrical stimulation, a natural environmental cue, is conducive to promoting conduction phenotypes [20]. Electrically pacing the CMs seeded on the collagen sponges in Matrigel promoted CM orientation [14, 32], which can be quantified as a physiological volume fraction ratio of myofilaments, mitochondria, and nuclei. This fraction ratio ranged from 10%:8%:27% in the unpaced constructs to 32%:10%:15% in the paced constructs in comparison with 40%:20%:5% in adult hearts. Electrical fields can be also applied to increase action potential duration and mitochondrial content; thereby enhancing the conduction velocity, cell orientation and contractile force [14, 32, 36, 45, 60, 61].

1) Bioreactors to Optimize Electrical Stimulation—The development of bioreactors enabled electrical stimulation that could be delivered to *in-vitro* engineered cells for investigations. Different parameters of electrical stimulation have been optimized, including frequency, pulse-width and amplitude, in the bioreactors [62]. Tandon *et al*. demonstrated systems which were capable of varying stimulation pulse configurations applied to a single cell plated on patterned substrates (Fig. 1A(a)), a monolayer of CMs on flat substrates (Fig. 1A(b)), or a 3-D cardiac tissue construct (Fig. 1A(c)) [48–50]. Microfluidic channels were developed with stimulation electrodes which could deliver varying pulsatile-electrical fields to enhance cell-cell coupling and synchronous contraction in both 2- and 3-D cardiac tissues [48–50]. By deploying a pulse with an amplitude of 3 V/cm and a frequency of 1 Hz, Tandon and her colleagues found the cultured neonatal rat CMs developed a high tissue density to exhibit synchronized contractility, accompanied by an increase in *Troponin-I* and *Cx43* expression [50].

Numerous laboratories have further investigated the impacts of electrical stimulation regimens for enhancing cardiac cell properties. Chronic applications of electrical pulses demonstrated the stabilization of action potentials of cultured neonatal rat CMs in both 2-D (1–3 Hz for 2–4 days) [44, 60] and 3-D cultured models (1 Hz for 8 to 9 days) [14]. The CMs underwent elongation in the direction of electrical field stimulation, exhibiting a contractility similar to the agematched native myocardium [45], in contrast to the disorganized sarcomeres in non-stimulated myocytes. The percentage of membrane with positive staining for *Cx43* and gap junction proteins was higher in comparison with the non-stimulated CMs [45]. Lasher *et al*. applied carbon rod-based stimulation electrodes to generate 2 ms symmetric biphasic square pulses at 4 V/cm and 1 Hz for 9 days, and demonstrated elongated CMs, accompanied by sarcomere alignment and *Cx43* up-regulation [45]. In their work, Lieu *et al*. applied cyclic electrical stimulations at 2.5 V/cm and 1 Hz for 14 days to the human embryonic stem cell (hESC)-derived CMs [36], and demonstrated increased amplitudes in potassium and calcium currents. Moreover, Nunes *et al*. delivered cyclic electrical stimulation of variable frequency at 1–6 Hz for the first 7 days, then at constant 1 Hz for 14 days, and demonstrated that realignment and conduction velocity of

hESC-CMs were dependent on the stimulation frequency [32]. Taken together, integrating the novel 3-D microfluidic constructs with monophasic or biphasic pulses promotes CMs towards mature phenotypes [48]. A summary of electrical stimulation approaches employed for CMs stimulation is presented in Table 1.

2) Molecular Mechanisms in Response to Electrical Stimulation—CMs derived from hESC have been well-recognized to display pro-arrhythmic action potential (AP) properties, including a high degree of automaticity, depolarized resting membrane potential, phase 4-depolarization, and delayed after-depolarization. While electrical stimulation has been implicated in regulation of ionic currents in ventricular myocytes [63], its role in maturation of action potentials in ESC-CMs and hESC-CMs remains to be investigated. At the molecular and cellular levels, re-orientation and elongation of CMs were completely abrogated by inhibiting actin polymerization with the use of Cytochalasin D, and partially by inhibiting phosphatidyl-inositol 3 kinase pathway by LY294002 [64]. However, the mechanisms underlying electrical pacing and CM realignment and excitability are still undefined.

3) Nanotechnologies to Enhance Electrical Stimulation—To facilitate syncytial electrial conduction, investigators have explored the use of nanostructures [47, 54]. When gold nanowires were embeded in the alginate scaffolds, Dvir *et al*. observed an improved synchronous contractilility among the adjacent CMs in response to electrical pacing. The nanowires (average length at ~1 μm and diameter at ~30 nm) were longer than the average thickness of the alginate pore wall (~500 nm); thus, allowing for electrical conduction and propagation throughout the scaffold. To address issues of nano-toxicity, Mooney *et al*. seeded mesenchymal stem cells (MSCs) on carbon nanotubes (CNTs)-based polylactic acid scaffolds in the culture medium containing CNTs [47]. The combination of CNTs and electrical stimulation up-regulated homeobox (*Nkx2.5*), transcript factor (*GATA-4*), cardiac *troponin T (CTT)* and *Cx43* protein expressions.

B. Mechanical Stimulation

Mechanical stress is also intimately linked with cytoskeletal architecture [13] as evidenced by a host of hydrogel-based studies [65, 66]. At the molecular level, mechanical forces induce chemical and electrical responses in CMs affecting cellular structure and function. For example, twitch forces of rat engineered heart tissue (EHT) were measured at a range of 2–4 mN/mm^2, while those of hESC-and hiPSC-constructs were at 0.08–0.12 mN/mm^2 in comparison with 40–80 mN/mm^2 in the intact heart muscle [15, 67, 68]. The lower forces of the engineered cardiac construct and ESC-CMs implicated a lower CM density and sarcomere volume fraction. However, introducing phasic mechanical stretches at 10% to 15% demonstrated an improved cardiac tissue structure and force development [43]. Discher's group further investigated matrix elasticity and fibrosis of stem cell-derived CMs in the presence of extracellular matrix (ECM) [55, 69]. In healthy myocardium CMs attach to a collagen-based ECM, generating sufficient compliance for actomyosin forces for myocardial contraction. The elasticity (*E*) of ECM was believed to influence cell shape, protein expression and organization, as well as differentiation. In the infarcted tissue, the scar engenders an increase in Young's modulus (E_{hard}=35–70 kPa) in comparison with that

of the native myocardium (11 kPa) [69]. Thus, both twitch forces and ECM elasticity are compliments to the mechanical phenotypes of hESC- and hiPSC-CMs.

1) Delivery of Mechanical Stimulation—Apparatuses have been developed to deliver strain to CMs [35, 42, 46, 70–72]. A static tension (strain) can be delivered to the engineered cardiac tissues between 2 glass rods or 2 steel needles [21, 73, 74]. Further implementation of mechanical stimulation has been made possible via uniaxial, biaxial or multiaxial stretching as illustrated in Fig. 2 [35, 42, 46, 70–72]. It has been showed that both static and cyclic uniaxial stress increased cell and matrix fiber alignment in hESC- and hiPSC-CM constructs [15]. Further, Fink *et al*. applied 20% uniaxial cyclic stretch at 1 Hz for 6 days to the neonatal rat and chick cardiac muscle, demonstrating CM alignment, elongation, and cardiac hypertrophy, accompanied by a 2- to 4-fold increase in contractile forces [43]. Akhyari and colleagues applied a 20% cyclic stretch at 1.3 Hz for 14 days to human neonatal heart cells, demonstrating enhanced cell spreading, distribution and proliferation [38]. However, a short-term 10% cyclic stretch at 1 Hz for 24 hours did not yield any effects on the neonatal CMs [39]. Kensah *et al*. further applied 10% cyclic stretch at 1 Hz for 7 days to the murine iPSC-CMs, demonstrating no changes in CM morphology. Nevertheless, applying static stretch (G-stretch) from day 7 to day 21 resulted in CM elongation [35], accompanied by uniform distribution of aligned sarcomeres. These indicated that rate, duration, frequency and type of mechanical stimulation are essential factors, requiring thorough investigations, in order to optimize a stretch regime for CM maturation, integration and alignment.

Recently, stretching followed by pacing to the engineered heart tissues successfully resulted in the contractile characteristics of native myocardium. These tissues possessed a high ratio of twitch (0.4 to 0.8 mN) to resting tension (0.1 to 0.3 mN) in response to β-adrenergic inotropic stimulation [19]. Briefly, isolated CMs from neonatal rats were cultured with collagen type I, basement membrane protein mixture (Matrigel), and concentrated serum-containing culture medium in a circular casting molds embedded with polytetrafluoroethylene (Teflon). After 7 days, the EHT was transferred into a stretch device for unidirectional and cyclic stretch (10%, 2 Hz). Next, the EHTs were transferred into thermostated organ baths (at 36°C) in which electrical field at 1 Hz was applied. Spontaneous contractions of EHT were observed when the stretch device was turned off in a culture dish [19], displaying cross-striation but at a lesser degree than in the adult tissue, along with a stable resting membrane potentials of −66 to −78 mV, fast upstroke kinetics, and a prominent plateau phase [19]. This suggested mechanical stimulation, namely stretching is capable of promoting the functional phenotypes of CMs.

To further demonstrate the interplay between vascular endothelial cells and CMs, Tulloch *et al*. showed an increase in proliferation under co-cultured human endothelial cells with iPSC-CMs in the presence of 5% cyclic stretch at 1 Hz for 4 days [15]. When the human ESC-CMs and iPSC-CMs were cultured on 3-D collagen matrices that were fixed at both ends to provide resistance against cell contraction, the CMs aligned in the direction of the applied resistance, accompanied by hypertrophy and proliferation [15]. Meanwhile, the vascular cells self-organized into vessel-like structures in the collagen/CM tissue, and the vessel

structures appeared to transport blood after one week of engraftment. In summary, a host of mechanical stimulation approaches is provided in Table 2.

2) Mechano-transduction Underlying CM Architecture—At the molecular level, protein complexes that sense mechanical stimuli are coupled to the cytoskeleton. While the specific mechano-sensing proteins remain to be identified, they are associated with specific cytoskeletal substructures, such as the Z-disc. The architecture of the cytoskeleton allows for mechano-sensitivity and mechano-transduction in response to the mechanical inputs [13, 75, 76], including cytoskeletal re-organization between mechano-sensitivity and structural remodeling.

The cytoskeleton is also associated with mechano-electrical coupling [77]. Mechano-sensitive cytoskeleton transmits mechanical forces to the ion channels with an implication in action potential duration. Galli *et al*. reported that microtubule depolymerizing agents increase the probability of L-type Ca^{2+} channels in a closed state, whereas microtubule-stabilizing agents increase the probability of an open state [78]. Furthermore, actin filament inhibitors affect the gating of K_{ATP} channels [79, 80]. Thus, the mechano-sensitive cytoskeleton is implicated in modulating ion channel activity and CM excitability.

C. Simultaneous Electrical and Mechanical Stimulation

Synchronizing stimulations may further facilitate the maturation of electrical and mechanical phenotypes of CMs. When Duverger *et al*. applied both electrical stimulation and uniaxial linear stretch to the cultured monolayer of CMs that were seeded on the polydimethylsiloxane (PDMS) template [42], they were able to induce transmembrane potential and calcium transient. In their work, Li *et al*. combined various electrical stimulation configurations (0.5, 1 and 2 V/cm at 1 Hz, 2 ms, and 30 minutes) and 10% tensile stretch to the cultured CMs isolated from neonatal Sprague-Dawley rats [46]. They demonstrated that *Cx43* mRNA was up-regulated to a higher extent at 0.5 V/m than at 1 and 2 V/cm. *Cx43* mRNA expression was also up-regulated in response to 10% tensile stimulation for 6 hours. Further, Maidhof *et al*. revealed the critical role of simultaneous electrical stimulation and medium perfusion to enhance contraction amplitude and mature CM phenotypes [72]. Similarly, the neonatal Sprague-Dawley rat CMs that were subjected to medium perfusion and continuous electrical stimulus (74.4 mA/cm², 2 ms, bipolar, 1 Hz) for 4 days displayed cell elongation, striation and up-regulation of *Cx43* [81].

III. MYOCARDIUM ASSESSMENT

While electrical and mechanical stimulations are capable of promoting CMs towards development, maturation and integration; validating the physiological phenotypes of regenerating myocardium is essential to address the outcomes of stimulation regimens and to provide feedbacks for therapies. In general, CMs and the beating heart could be electrically and/or mechanically accessed to obtain morphological information, contractile performance, electrophysiological properties and excitation-contraction coupling (ECC) [37]. Imaging techniques have been commonly used to assess the cell-based morphology and Ca^{2+} transients [56, 82, 83]. Gepstein *et al*. used optical mapping to reveal the regional and global effects in response to hESC-CM transplantation [56]. Although the transplanted

cell seems to integrate electrically, localized conduction delays and blocks were observed. Magnetic resonance imaging (MRI) has been employed to investigate the left ventricle (LV) performance after the transplantation of ESC-CMs [84], and to study myocardial contractility after injecting the hESC microvascular grafts embedded in the synthetic hydrogels to the rat model of myocardial infarction [85]. The MRI approach revealed arrhythmogenicity associated with altered action potential duration, and depolarized resting membrane potential.

A. Electrical Strategies

Electrical contraction of the mammalian hearts at the cellular and molecular levels is recorded as action potentials from the individual CMs (Fig. 3a). The sequential activation starts in the sinoatrial (SA) node, then propagates through the atria to the atrioventicular (AV) node, and the electrical activity spreads out to excite the ventricular myocardium via the bundle of His and conducting Purkinje fibers [86]. The cardiac action potentials are generated by the sequential activation and inactivation of ion channels conducting depolarizing and repolarizing currents as shown in Fig. 3 (a).

Voltage-clamp technique initiated by Cole *et al*. in the 1960s [87–90] has been further developed and widely used to characterize ion channel currents in myocardial cells in time- and voltage-dependent fashion, providing insights into ion permeability, selectivity, and reversal potentials [86]. Malan *et al*. investigated the long-QT syndromes (LQTS) using iPSC-CMs in a mouse model. Voltage clamp allowed for the assessment of the biophysical effects of the mutation of the Na^+ channels, revealing a faster recovery from inactivation and a higher late current as compared to those of the control [91]. Patch clamp further allowed for the use of ESC- and iPSC-CMs to model cardiac diseases, revealing that the mutants have a significant decrease in I_{Na} density but larger persistent I_{Na} current as compared with the wild-type CMs [92]. Lieu *et al*. investigated the underlying mechanisms to maturate human ESC and iPSC-CMs by providing environmental cues. They found that a lack of the Kir2.1-encoded inwardly rectifying K^+ current (I_{K1}) is the underlying mechanistic contributor to the immature electrophysiological properties among the panoply of sarcolemmal ionic currents investigated ($I_{Na+}/I_{CaL}{}^{2+}/I_{Kr}{}^{+}/I_{NCX}{}^{+}/I_f{}^{+}/I_{to}{}^{+}/I_{K1}{}^{-}/I_{Ks}{}^{-}$) in hESC-CMs [36]. Their findings provided a basis to develop a biomimetic culturing strategy for enhancing maturation. Recently, patch-clamp recordings have been employed to verify the functionality and maturity of ESC- and iPSC-CMs in response to drug and toxicity screening [36, 91–93]. The electrical phenotype of myocardium was also analyzed via the assessment of cardiac action potentials as reported in numerous papers [32, 36, 52, 60, 93]. The information incorporated in the cardiac action potentials such as slopes, duration and waveform could be elucidated for assessing cell functionality and maturity for the cardiac cell-based therapy [1, 32, 36].

In corollary, studying the global electrical phenotypes of the regenerating myocardium to provide feedbacks is complementary to successful cell-based transplantation. In humans, the 12-lead electrocardiogram (ECG) has been routinely used to detect myocardial ischemia, infarction, and arrhythmia. In the small animal models of heart regeneration, ECG signals from both adult and embryonic zebrafish as well as neonatal mice have provided the distinct

P wave and QRS complex resembling that of humans (Fig. 3 (b)) [6, 7, 57, 58, 94–96]. Using the wavelet transform for signal processing [57, 58, 94–96], we revealed that ventricular repolarization (ST intervals and T waves) failed to normalize despite fully regenerated myocardium at 60 days post ventricular amputation, suggesting further cardiac remodeling may be required to fully integrate regenerating myocardium with host myocardium. Thus, these findings suggested the need to elucidate mechanisms underlying electrical conduction and heart regeneration. The advances of flexible electronics have enabled the application of polymer-based microelectrode array platform for the small animal models of heart regeneration [58, 94, 96]. The MEA membranes were micro-fabricated with the gold electrodes embedded in parylene C to adhere to the non-planar surface for the detection of electrical conduction. The acquired ECG signals were processed to provide long-term monitoring of electrical phenotypes with a high signal-to-noise-ratio [58, 94, 96].

Integrating ECG with the genetically encoded calcium sensor (GCaMP3) fluorescent signals, Shibba *et al*. assessed the activity of hESC-CM grafts in the injured hearts of a guinea-pig model in order to investigate the electromechanical integration and risk of arrhythmias after transplantation. To conduct the study, they performed cryoinjury and treated the animals with 1×10^8 hESC-CMs in a pro-survival cocktail (PSC), 1×10^8 non-cardiac hESC-derivatives in PSC and PSC alone. According to their observations, they have successfully demonstrated that the group with hESC-CM grafts displayed lower risk of ventricular tachycardia and arrhythmias compared with the other two [30]. The ECG and fluorescent data also confirmed the improvement in integration and synchronization with the host of the hESC-CM group.

B. Mechanical Strategies

The pacemaker cells in SA and AV nodes provide a coordinated electrical propagation to initiate syncytial cardiac contraction. In addition to the electrical phenotypes, the mechanical phenotype, namely myocardial contractile force, has an impact on the functionalities of CMs and cardiac tissue. Since the successful demonstration of their first EHT model in 1997, Eschenhagen *et al*. has applied a force transducer using a thermal array to measure the contractile force delivered by the CM construct [21]. Similar approaches have been proposed, developed and utilized to acquire the contractile force of a single CM, a cell sheet, or a 3-D cardiac tissue construct [12, 26, 38, 39, 43, 97–100] as illustrated in Fig. 4.

Sivaramakrishnan *et al*. deployed coupled carbon nanofibers to form a piezo-translator for acquiring the contractile force of a seeded CM and for determining the β-cardiac myosin function in response to CM contraction [98]. This approach can be further utilized to study the genetic and phenotypic impacts of environmental cues to a single cell. To verify their method of using cell sheets, Shimizu and his colleagues employed a commercially available strain gauge integrated in a 4-sheet graft transplanted to a rat heart [26], and were able to measure a contractile force of 1.18±0.26 mN (n=3) after 3 weeks of transplantation. This *in-situ* implementation suggested the feasibility of integrating a force measurement to investigate cardiac grafts. Park *et al*. further designed a hybrid biopolymer cantilever beam on which CMs can be seeded for measuring the contractile force [101]. This method utilized a flexible and transparent substrate, thus enabling the correlation with other optical

approaches as well as the integration with mechanical stimulations by stretching the polymer. Recently, Liu *et al*. employed the atomic force microscopy (AFM) to quantify the mechano-biological properties of iPSC-CMs and ESC-CMs in terms of contractile force, rate, duration, and cellular elasticity. The use of AFM to assess the contractility of CMs is in need of synchronizing the z-piezo of the AFM with contracting CMs [102, 103], which introduces fluidic disturbances, rendering the measurements inaccurate. Different from previous approaches using AFM, Liu and colleagues made a gentle contact between the AFM cantilever and CMs, followed by locking the z-piezo, allowing the contractile CMs to deflect the cantilever beam. The deflection was then used to calibrate the force with the spring constant with an improved sensitivity and spatial resolution. Thus, their approach showed potentials to further assess mechanical properties of a single CM for elucidation.

At the organ level, high-frequency ultrasonic transducers have provided an entry point to unravel the micro-structures of small animal hearts and pulsed-wave (PW) Doppler ultrasound allows for real-time blood flow measurements [104–106]. While high frame rate ultrasound biomicroscopy (UBM) provides images with high spatial and temporal resolution, PW spectral Doppler reveals the direction and magnitude of velocity profiles. The ratios of E- to A-waves reflect the ventricular compliance or clinically known as diastolic function. Thus, high-frequency ultrasonic transducers hold promises for a real-time and non-invasive strategy to assess myocardial compliance.

C. Synchronized Electrocardiogram and Echocardiogram to Access Myocardium

To further investigate whether cell-based therapy restores cardiac function, one can develop a simultaneous ECG and PW Doppler for the long-term monitoring of electrical and mechanical coupling in injured and regenerating hearts. While micro-ECG (μECG) provides electrical phenotypes, PW Doppler signals reveal the mechanical and physiological insights into myocardial compliance and ventricular function. The use of zebrafish (*Danio rerio*) has further provided a model for the proof-of-concept. Unlike adult mammalian tissues, zebrafish and some certain amphibians maintain a regenerative capacity throughout adult life. Zebrafish hearts fully regenerate after 20% of ventricular resection [8], thereby providing a genetically tractable system for drug discoveries and inherited cardiac arrhythmias [96, 107].

Simultaneous ECG-gated echocardiogram and PW Doppler has further improved signal acquisition to validate the electrical and mechanical phenotypes (Fig. 5). The presence of P wave and QRS complexes were superimposed with the PW Doppler-E- and A-waves to assess the inflow profiles from atrium to ventricle. While E waves represent passive filling of the ventricle, A waves reflect the active filling in response to atrial systole. In humans, the E-wave velocity is greater than that of the A wave (E >A). In zebrafish, E/A reversal is observed, suggesting a distinct physiology in the two chamber system in which atrial systole generates high ventricular filling pressure in the setting of the highly trabeculated ventricle. Together, simultaneous ECG and PW Doppler strategy provide a minimally-invasive approach to assess electrical and mechanical coupling and ventricular compliance in animal models of heart regeneration.

IV. DISCUSSIONS

Advances in differentiation of hPSCs into functional CMs have provided a platform for regenerative medicine, drug screening and toxicity testing [37]. However, translational benefits have been hampered by the immature properties of these hPSC-CMs in cell size, length-to-width ratio, mitochondrial quantity, morphology, and appearance of T-tubules [37]. In the immature hPSC-CMs, the upstroke velocity and resting membrane potential are ~50 V/s and ~−60 mV, respectively, in contrast to 250 V/s and ~−90 mV in adult cells [37]. Similarly, conduction velocity is ~0.1 m/s in comparison to 0.3–1.0 m/s in adult cells, and membrane capacitance is 17.5±7.6 pF in comparison with that ~150 pF in adult CMs [37]. Thus cultivating the maturation of the hPSC-CMs requires a combinative approach, including long-term culture, 3-D tissue construction, mechanical loading, electric stimulation, substrate stiffness, and neuro-hormonal factors [37]. Although transplanting human ESC-CMs to the periinfarcted regions has been reported to improve the ventricular ejection fraction, the risk of arrhythmias following hESC-CM transplantation remained unresolved [30]. While priming the hESC-CMs with electrical and mechanical stimulation promoted has been successful in synchronous contractions of cultured cardiac constructs, realignment of sarcomeres, induction of striations and gap junctions [17, 20, 37, 108], the integration of ESC-CMs and iPSC-CMs to an injured site still confronts unmet bioengineering challenges.

Rapid advances in cardiac tissue engineering based on animal models such as zebrafish or neonatal mice [6–9] have provided new insights into cell-based therapy [10–12]. Neonatal rat heart cells have been widely used for their resistance against hypoxia [20, 109]. However, *in-vitro* and animal models have encountered translational roadblock to pre-clinical trials. One of the contributing reasons is that heart tissue heterogeneity, in terms of proportion of CMs to non-myocytes (fibroblasts, endothelial and smooth muscle cells), varies from 20% in humans to 50% in mice [110, 111]. The presence of non-myocytes and micro-environmental factors, impacts the repair and function of a healthy heart, and thus, the success of cell-based therapy [15, 35, 112].

Incorporating the environmental cues holds promise to promote cell-based therapy. Topographic cues are conducive to stretch-mediated CM functionality. Micro-environments, including ECM, neighboring cell-cell interactions, substrate stiffness and topology, contribute to the spatial distribution of *Cx43*, uniaxial alignment of sarcomeres, up-regulation of adherent junction (*N-cadherin*) [113], and activity of protein kinase C [40]. Both actin polymerization and cytoskeleton are implicated in sarcomere alignment. The cytoskeleton is also essential in propagating mechanical stimuli to mediate function within the CMs, including signaling, excitability, electrical propagation, contractility and protein expression. While the precise mechanism remains unknown, the expression and orientation of mechano-sensors are implicated in the adaptability of CM to mechanical loads. Extracellular stiffness close to that of native myocardium (10–11 kPa) is recognized to enhance CM maturation as reflected by aligned sarcomeres, mechanical forces and calcium transients and sarco/endoplasmic reticulum calcium-ATPase 2a expression [55, 69, 114].

Overall, electrical and mechanical strategies promote CM maturation and functionality as well as provide feedbacks for cardiac repair and heart regeneration studies. Their relative and collective contributions reside in the micro-environmental cues. However, in order to thoroughly investigate the underlying mechanisms of electrical and mechanical cues on the developmental properties of CMs, numerous variables need to be considered since the 3D *in-vivo* CMs function in contact with other cells (fibroblasts, smooth muscle and other cells) and the iPSC-CMs may contain mutations that affect mechanical and electrical properties [115].

With the recent development of flexible and stretchable micro/nanotechnologies [58, 94, 116, 117], close-loop systems to simultaneously "train and screen" ESC- and iPSC-CMs are of interest. Microfluidic-based bioreactors which offer both "training and screening" functions are also conducive to cardiac drug discovery and screening [52, 118]. Thus, developing non-invasive and real-time methodologies will further enable us to monitor integration of cell-based therapy.

V. CONCLUSION

Over the past decade, engineering 3-D heart muscles has made a significant progress, standardizing human myocyte production protocols. However, establishing effective but simple strategies for the *in-vitro* vascularization of 3-D constructs, maturation of myocytes, and defining the predictive indices for preclinical safety remain critical to address tissue engineering for cardiac repair [20]. Aristotle stated:

> ***The search for truth is one way hard another easy, for no one can master it fully nor miss it fully, each adds a little knowledge to our nature, and from all things assembled there arises a certain grandeur***.

In this context, the central theme of this review paper highlighted the interdisciplinary efforts to promote CM maturation for integration with the host myocardium as well as to provide feedbacks via phenotype assessment for diagnosis, prognosis and monitoring of injured and regenerating hearts.

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K. Kirk Shung obtained a B.S. in electrical engineering from Cheng-Kung University in Taiwan in 1968, a M.S. in electrical engineering from University of Missouri, Columbia, MO in 1970 and a Ph.D. in electrical engineering from University of Washington, Seattle, WA, in 1975. He became an assistant professor at the Bioengineering Program, Pennsylvania State University, University Park, PA in 1979 and was promoted to professor in 1989. He was a Distinguished Professor of Bioengineering at Penn State until 2002 when he joined the Department of Biomedical Engineering, University of Southern California, Los Angeles, CA, as a professor. He became a Dean's professor in biomedical engineering, an endowed position, in 2013. He has been the director of NIH Resource on Medical Ultrasonic Transducer Technology since 1997.

Dr. Shung is a life fellow of IEEE, and a fellow of the Acoustical Society of America and American Institute of Ultrasound in Medicine. He is a founding fellow of American Institute of Medical and Biological Engineering. He has served for two terms as a member of the NIH Diagnostic Radiology Study Section. He received the IEEE Engineering in Medicine and Biology Society Early Career Award in 1985 and was the coauthor of a paper that received the best paper award for IEEE Transactions on Ultrasonics, Ferroelectrics and Frequency Control (UFFC) in 2000. He was selected as the distinguished lecturer for the IEEE UFFC society for 2002–2003. He was elected an outstanding alumnus of Cheng-Kung University in Taiwan in 2001. In 2010 and 2011, he received the Holmes Pioneer Award in Basic Science from American Institute of Ultrasound in Medicine and the academic career achievement award from the IEEE Engineering in Medicine and Biology Society.

Dr. Shung has published more than 500 papers and book chapters. He is the author of a textbook "Principles of Medical Imaging" published by Academic Press in 1992 and a textbook "Diagnostic Ultrasound: Imaging and Blood Flow Measurements" published by CRC press in 2005. He co-edited a book "Ultrasonic Scattering by Biological Tissues" published by CRC Press in 1993. Dr. Shung is currently serving as an associate editor of IEEE Transactions on Ultrasonics, Ferroelectrics and Frequency Control, IEEE Transactions on Biomedical and Engineering, and Medical Physics. Dr. Shung's research interest is in ultrasonic transducers, high frequency ultrasonic imaging, ultrasound microbeam, and ultrasonic scattering in tissues.

Tzung Hsiai, MD, PhD, received an undergraduate degree with honor with distinction in Bioengineering from Columbia University, followed by his Doctor of Medicine from the University of Chicago, Pritzker School of Medicine. He subsequently performed an internship and residency in Internal Medicine at the Harbor-UCLA Medical Center, and Cardiology STAR Fellow at UCLA Medical Center and PhD in Biomedical Engineering at School of Engineering and Applied Science. As a fellow, he was awarded the UCLA Johnson Memorial Scholarship and Chancellor's Funds for Academic Border Crossing.

After his fellowship, Dr. Hsiai was recruited to University of Southern California as Robert G. and Mary G. Lane Early Career Chair, and was awarded Distinguished Young Alumnus Award by UCLA School of Engineering & Applied Science, USC Viterbi School of Engineering Junior Faculty Research Award, and AHA John J. Sampson Outstanding Research Award. Dr. Hsiai currently serves as Professor of Medicine and Bioengineering at the David Geffen School of Medicine and Henry Samuelli School of Engineering at UCLA in the Divisions of Cardiology and Department of Bioengineering. He is a Visiting Associate of Biomedical Engineering at California Institute of Technology. He is also directing the UCLA Cardiovascular Engineering Research and Laser Light-Sheet Imaging at the Center for Health Science. Dr. Hsiai is a member of the American Heart Association Scientific Sessions Program (CSSP) Committee of Atherosclerosis, Thrombosis, and Vascular Biology (ATVB) Council, Biomedical Engineering Society, American Association for Advancement of Science, American Physiologic Society, and certified by the American Boards of Internal Medicine and Cardiovascular Disease. He has been elected as a Fellow of the American Heart Association (Basic Cardiovascular Sciences Council), American College of Cardiology, and a member of American Society for Clinical Investigation. Dr. Hsiai is also a member of National Institutes of Health Bioengineering Technology and Surgical Science Study Section. Currently, he serves as a member of the Leadership Committee for the Cardiovascular Theme for UCLA David Geffen School of Medicine, and Legislative Assembly Representative for the Department of Medicine.

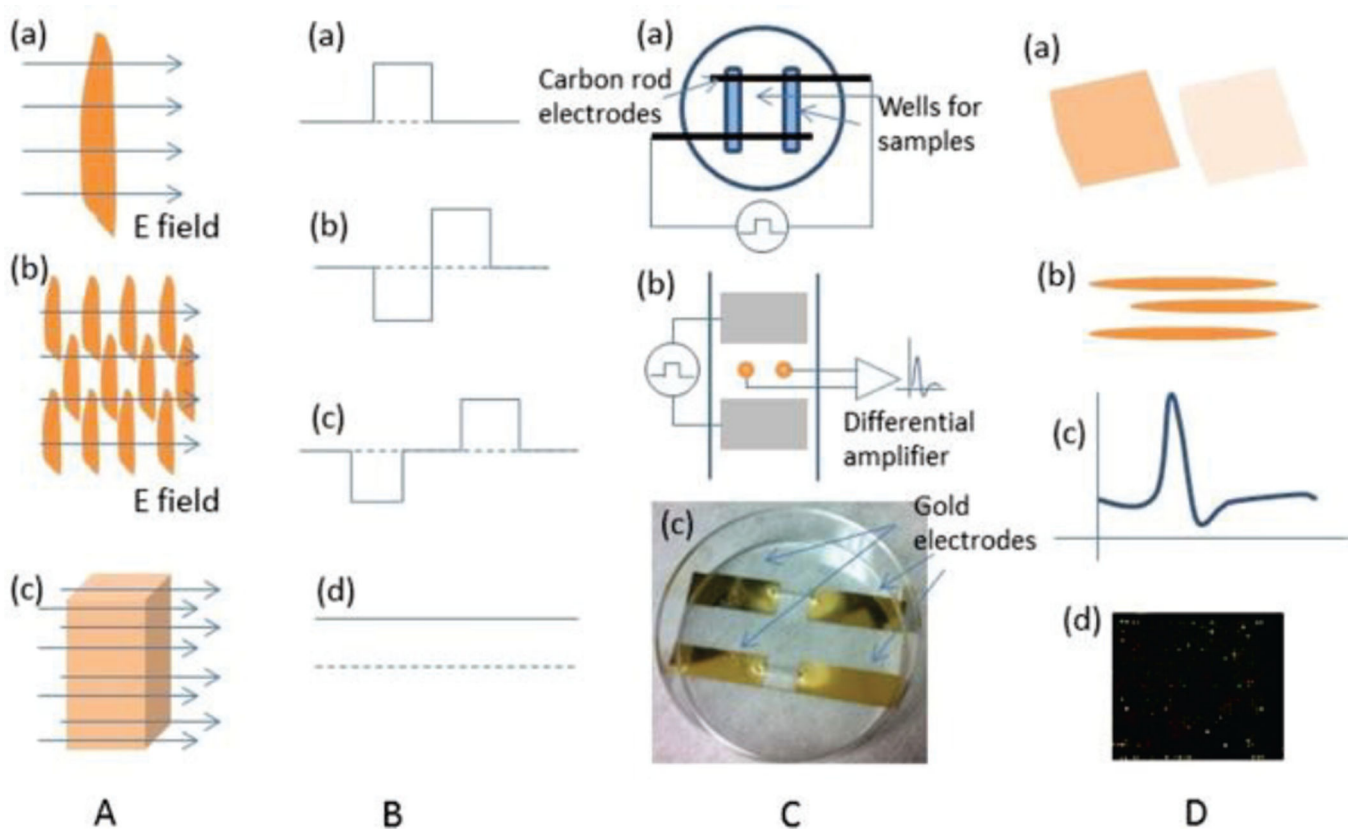

Figure 1. Electrical stimulation

A. The electrical filed stimulation was delivered to (a) a single cell, (b) a monolayer, and (c) tissue. **B**. Variations in electrical stimulation include (a) monophasic pulses, (b) charge-balanced biphasic pulses, (c) charge-balanced biphasic pulses with interphase delay, and (d) direct current. **C**. The design of microfluidic bioreactors includes (a) a Petri-dish with carbon rod electrodes and wells, (b) a customized screening apparatus with stimulation and recording electrodes, and (c) wells and gold stimulation electrodes. **D**. Assessment of cardiomyocytes demonstrates (a) contractile activity, (b) elongation and alignment, (c) electrical activity, and (d) gene expression.

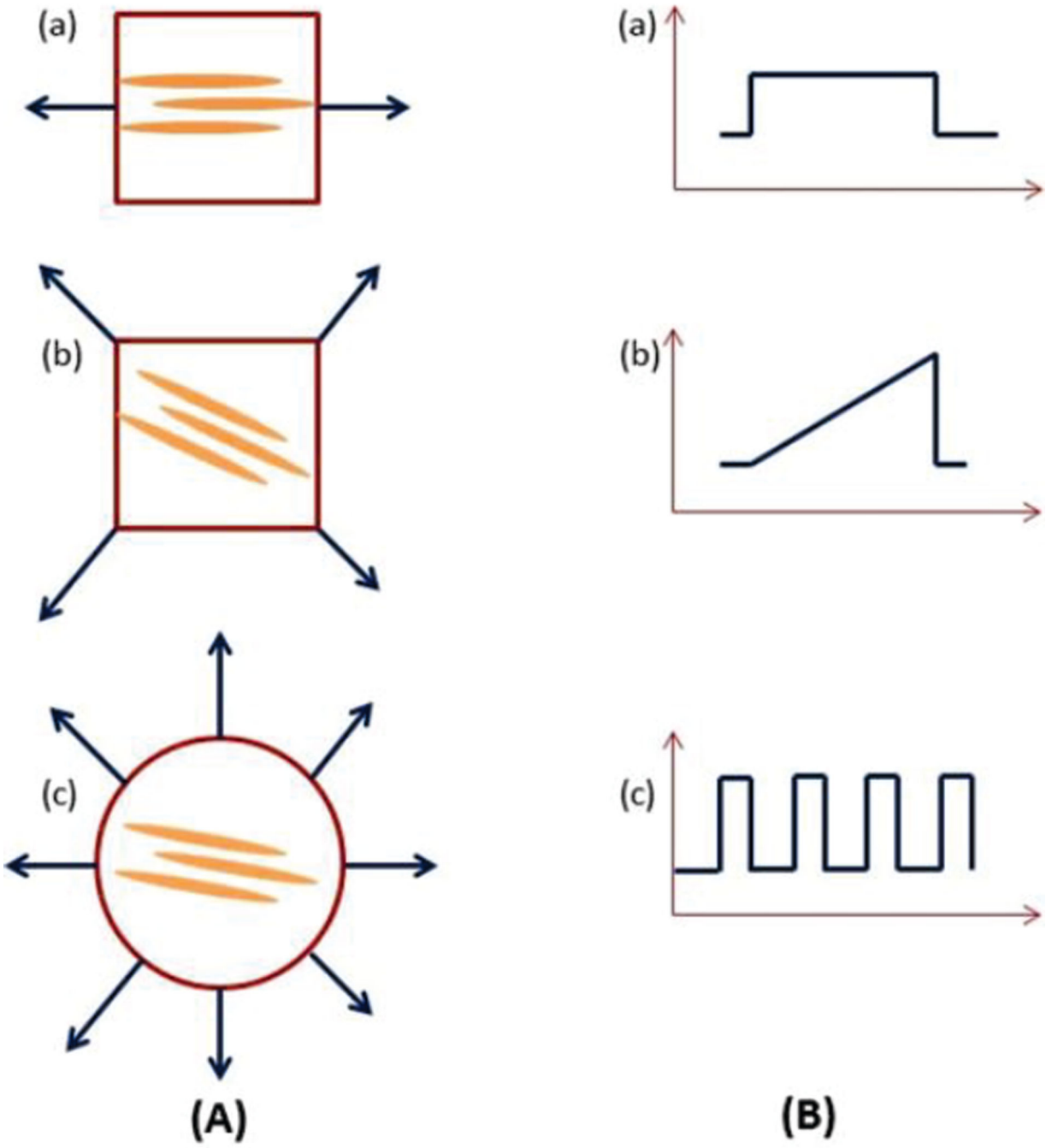

Figure 2. Mechanical stimulation

A. Mechanical stretches were implemented in (a) uniaxial, (b) biaxial and (c) multiaxial direction. B. Mechanical stretches over time were demonstrated in (a) static, (b) ramping and (c) cyclic manner.

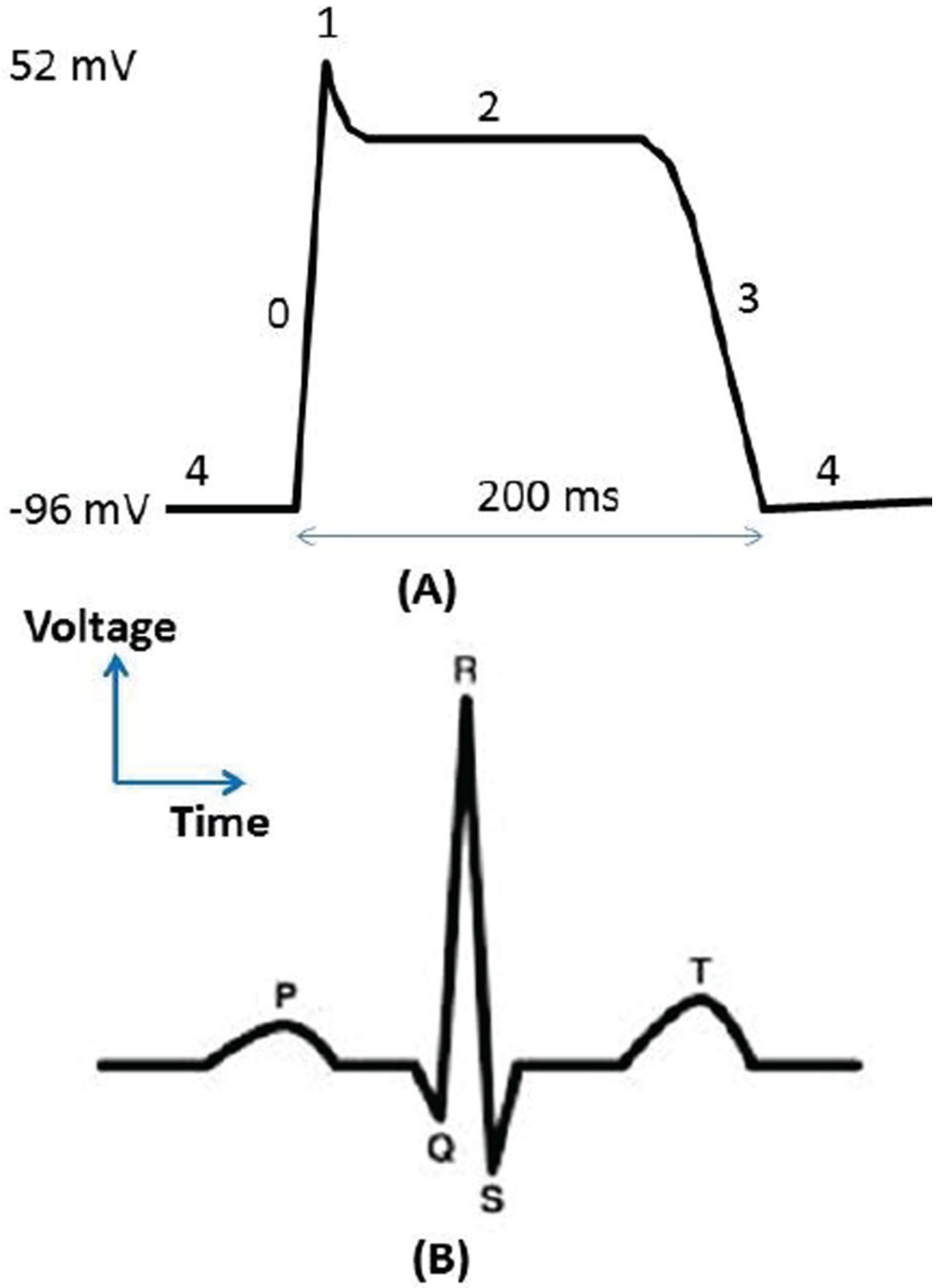

Figure 3. Electrical phenotypes

A. Cardiac action potential represents the distinct phases: phase 0 denotes the rapid depolarization during which Na+ ions enter into the cell rapidly, phase 1 involves the inactivation of Na+ channels while K+ and Cl− ions leave the cells, phase 2 involves a balance of Ca2+ (in) and K+ (out) currents, phase 3 includes a rapid repolarization during which Ca2+ channels close, K+ ions leave the cells, and phase 4 denotes the resting membrane potential, associated with diastole. B. Electrocardiogram displaying P waves (atrial depolarization), QRS complexes (rapid depolarization of ventricles) and T waves

(repolarization of ventricles). Other important properties are RR interval, ST segment and QT interval.

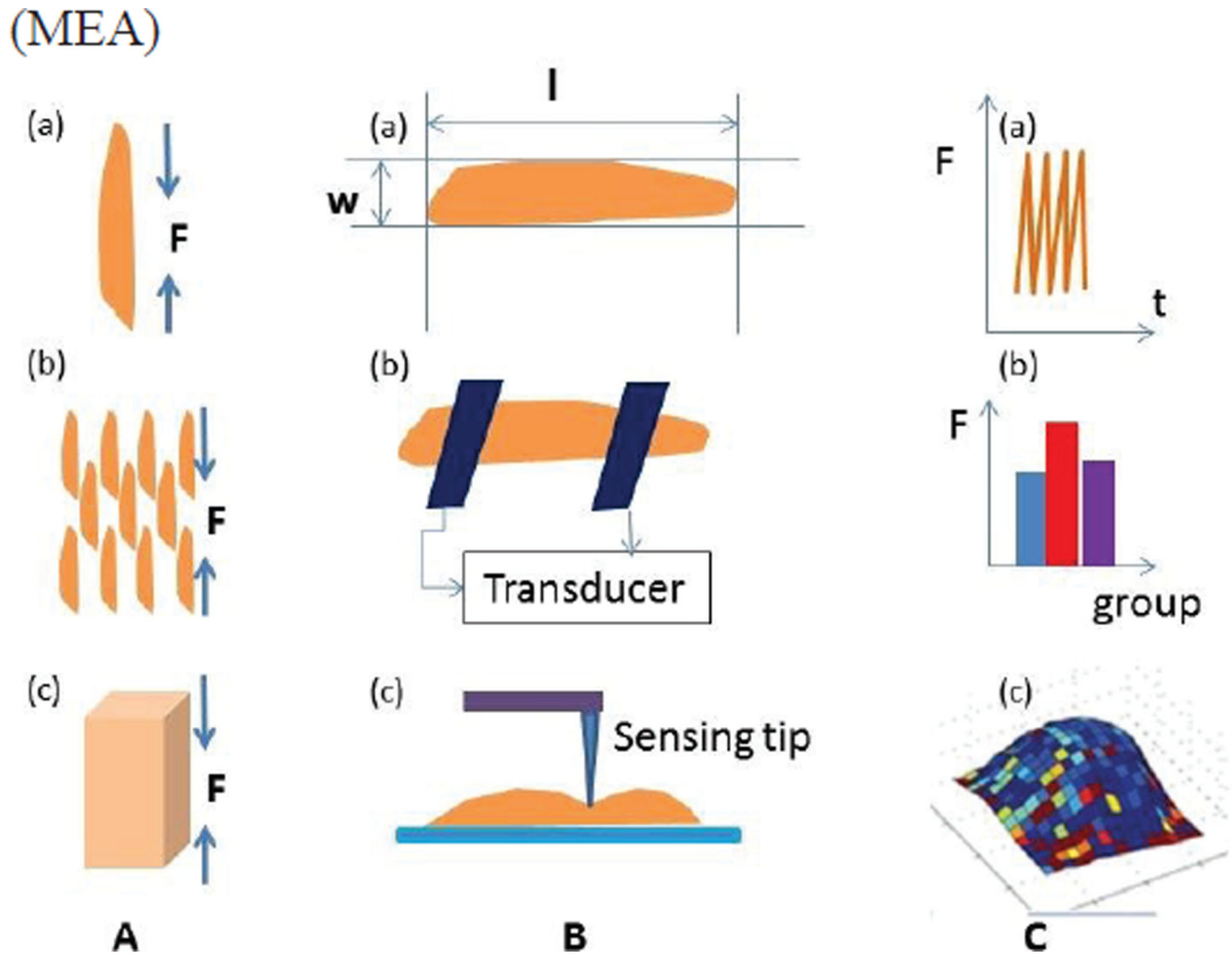

Figure 4. Mechanical properties assessment

A. The contractile force measurement can be applied for a single cell (a), a sheet of cells (b) and a 3D construct (c). B. The assessment can be performed by (a) acquiring geometric properties (length l and width w) to derive force, (b) applying piezoresistive or capacitive-based transducers or (c) using a sensing tip to transfer the mechanical property. C. The contractile force can be measured over time to quantify the amplitude and frequency (a), to compare among different groups of myocardium (b), or to observe the force distribution.

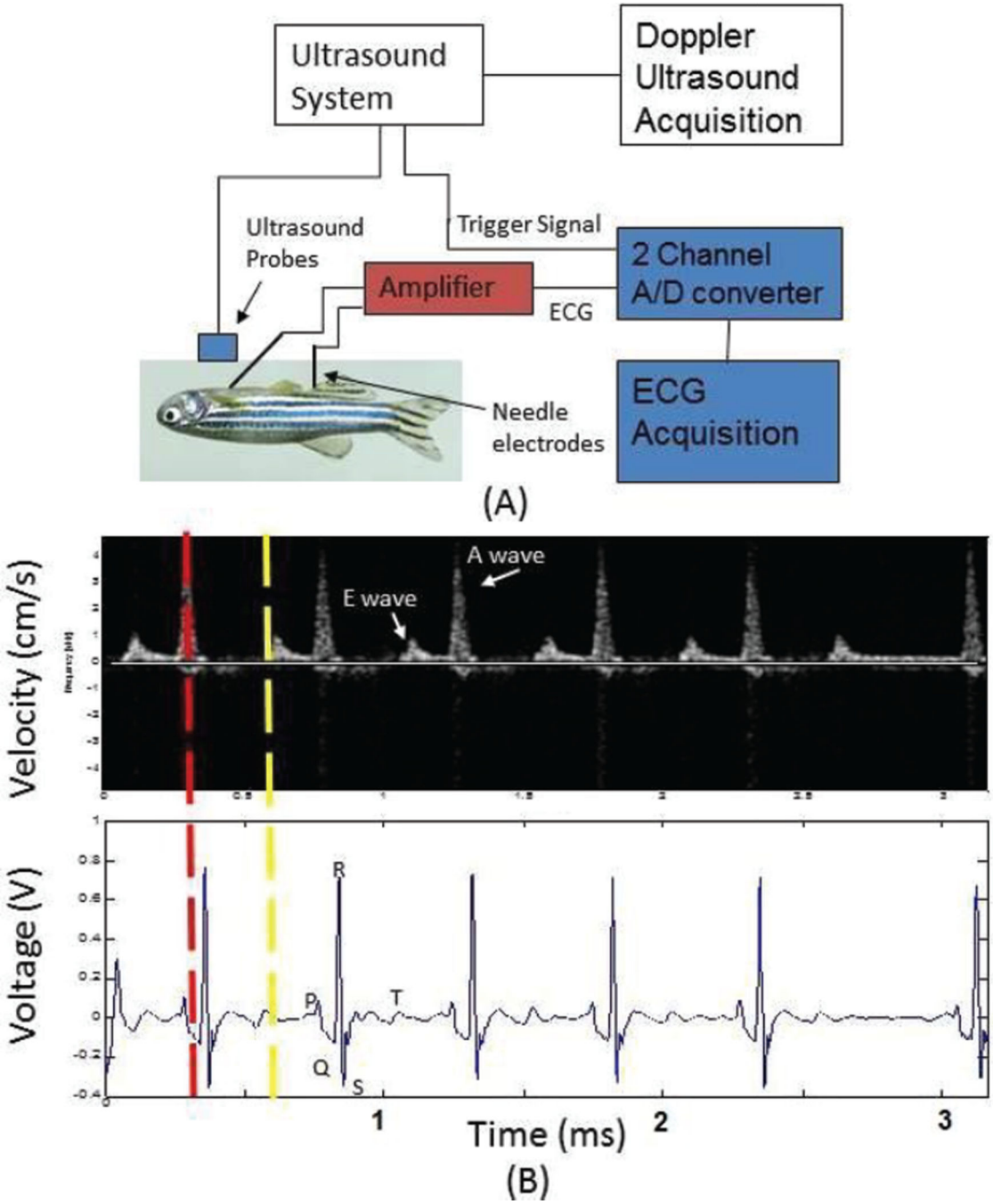

Figure 5. Synchronized PW Doppler ultrasound and ECG recording from zebrafish

A. Experimental setup for synchronized Doppler-ECG measurement. Adult zebrafish was sedated and placed with the ventral side facing up in a container filled with 0.01% Tricaine. A small opening to the chest exposes the heart. The linear array ultrasonic transducer was placed approximately 6 mm directly above the heart, and a needle electrode for ECG recording was introduced to the heart from lateral direction. A second needle electrode as a reference was placed towards the tail of the zebrafish. When the Doppler ultrasound recording starts, the ultrasound system sends trigger signals to initiate ECG recording, thus

allowing for a synchronized Doppler-ECG recording. B. Synchronized Doppler spectrum of ventricular inflow and ECG signals. The P wave in ECG precedes atrial contraction, which is corresponding to the A wave in Doppler; and T wave in ECG precedes ventricular relaxation, which is corresponding to the E wave in Doppler. There is a delay between electrical and mechanical coupling at approximately 50 ms.

TABLE I

Summary of electrical stimulation strategies

Cell types	Configurations	Duration	Results	Ref.
Neonatal rat CMs	2 ms, 5 V/cm, 1 Hz	8 days	Promoted alignment, contraction and excitability	[14]
Neonatal rat CMs	2 ms, 4 V/cm, 1 Hz	9 days	Enhanced elongation, CM volume fraction and *Cx43* expression	[45]
Neonatal rat CMs	2 ms, 3 V/cm, 3 Hz	5 days	Enhanced troponin-I and *Cx43* expression. Developed contractile behavior	[50]
Human ESC-CMs	2.5 V/cm, 1 Hz	14 days	Enhanced sarcomere organization and electrical properties	[36]
Human ESC- and iPSC-CMs	3–4 V/cm, 1–6 Hz for 7 days then 1 Hz for 14 days	21 days	Promoted alignment, Ca^{2+} handling and conduction velocity	[32]
Human MSC	2 ms, 0.15 V/cm, 1 Hz	14 days	Promoted alignment, elongation, protein expression	[47]
Neonatal rat CMs	4 ms, 2.5 V/cm, 3 Hz	6 days	Promoted action potential, conduction velocity	[60]
Neonatal rat CMs	10 ms, 80 V, 5 Hz	3 days	Increased in mRNA of contractile protein (3-myosin	[61]

TABLE II

Summary of mechanical stimulation strategies

Cell types	Configurations	Duration	Results	Ref.
Human pediatric CMs	1.34 Hz cyclic, 20% strain	14 days	Enhanced proliferation, distribution and mechanical strength	[38]
hESC- and hiPSC-CMs	1 Hz cyclic, 5% strain	4 days	Enhanced proliferation, hypertrophy	[15]
Neonatal rat CMs	1.5 Hz cyclic, 20% strain	6 days	Hypertrophy, enhanced contractile function	[43]
Mouse and hiPSC CMs	Static ramp with +2% after 2nd day	14 days	Hypertrophy, enhanced contractile function	[35]
Neonatal rat CMs	1 Hz, 15% strain	4 days	Induced striation, promoted *Cx43*	[70]
Neonatal rat CMs	1 Hz, 20% strain	6 days	Promoted alignment and elongation and *Cx43*	[71]