Application Note

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Using the Agilent xCELLigence RTCA ePacer for Functional Maturation of Human-Induced Pluripotent Stem Cell-Derived Cardiomyocytes

Authors

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Abstract

This application note presents a method for improving human-induced pluripotent stem cell-derived cardiomyocyte (hiPSC-CM) maturation. The Agilent xCELLigence RTCA ePacer system was used with microplates featuring planar interdigitated gold microelectrodes for directed electrical field stimulation of hiPSC-CMs. The resulting positive force-frequency relations and appropriate contractile responses to inotropic compounds show that extended electrical pacing leads to improved functional maturation of cardiomyocytes.

Introduction

One of the best-studied and characterized cell types generated from human-induced pluripotent stem cells are hiPSC-CMs, which are currently being used for drug discovery, toxicology, and investigating the underlying mechanism of cardiac disease.[1,2] These cardiomyocytes have already been implemented as an integral *in vitro* model for toxicology and safety assessment of pharmaceutical compounds under development,[3,4] particularly for assessment of arrhythmia.[5,6] hiPSC-CMs display the appropriate repertoire of ion channels and currents found in human adult cardiomyocytes. Numerous studies and approaches, including patch clamp recording, field potential (FP) recording, calcium transients, impedance (IMP) measurements, and vector motion analysis have been used to validate hiPSC-CMs as a predictive model system for safety and toxicology assessment.[7–10] The FDA has recently launched the Comprehensive *In Vitro* Pro-Arrhythmia Assay (CiPA) initiative to further explore and validate hiPSC-CMs for pro-arrhythmic assessment of compound risk with the aim of modifying the current regulatory guidelines for cardiac safety assessment to include hiPSC-CMs.[11,12]

One of the main obstacles to full implementation of hiPSC-CMs for drug discovery purposes, especially those drugs that may serve to modulate the force of cardiomyocyte contraction (inotropes), has been its inherently less developed or immature phenotype.[13,14] It is well known that the inherent structural hallmarks, calcium handling mechanism, electrophysiology, and gene expression profile of hiPSC-CMs resemble fetal rather than adult cardiomyocytes.[15–18]

From a functional perspective, hiPSC-CMs and human embryonic stem cell-derived cardiomyocytes (hESC-CMs) display a negative force-frequency relationship (NFF), whereas adult cardiomyocytes have an inherent positive force-frequency relationship (PFF).[15,18,19] Furthermore, hiPSC-CMs display rounded and multiangular morphology, and lack the characteristic rectangular morphology of adult cardiomyocytes, which is optimized for force generation and promotion of excitation-contraction coupling.[16,20,21] Various *in vitro* approaches, including long-term cell culture, growth factor stimulation, physical or mechanical stimulation, substrate stiffness, and electrical stimulation have been described to improve the maturation status of hiPSC-CMs.[22–27]

The maturation of cardiomyocytes can be defined from multiple aspects including structural, metabolic, electrophysiological, and functional maturation. The scope of this study is to improve hiPSC-CM maturation and evaluate it only at the functional level, specifically contractile activity of cells. To improve the maturation status of hiPSC-CMs, we have used the xCELLigence RTCA ePacer system (Figure 1A) in conjunction with microplates fabricated with planar interdigitated gold microelectrodes to perform directed electrical field stimulation of hiPSC-CMs (Figure 1B). The frequency of the applied electrical field was progressively increased, and contractility of cardiomyocytes was monitored by impedance measurement. The data demonstrate that cardiomyocytes subjected to prolonged electrical pacing display enhanced functional maturation of cardiomyocytes indicated by positive force-frequency relations and appropriate contractile responses to inotropic compounds.

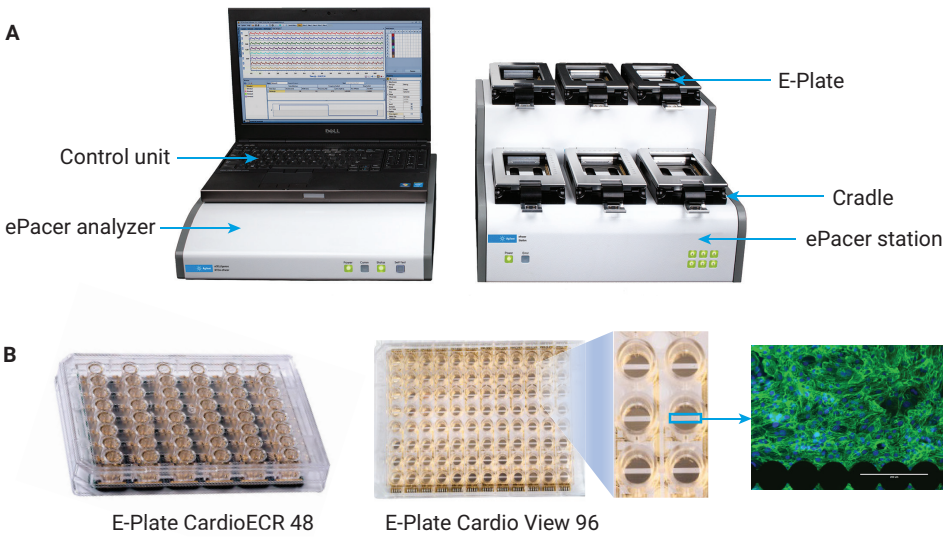


Figure 1. (A) The Agilent xCELLigence RTCA ePacer is a high-throughput 6-cradle platform that consists of four components: laptop, ePacer analyzer (left), ePacer station (right), and E-Plates. (B) An image of the Agilent E-Plate CardioECR 48 and E-Plate Cardio View 96. On the E-Plate Cardio View 96, a close-up of the wells reveals the layout of the electrodes as well as the "view area", an electrode-free gap that enables users to visually observe the cardiomyocytes in the wells.

Experimental

Materials and methods

Cell culture

hiPSC-CMs from FUJIFILM Cellular Dynamics International (FCDI/iCell cardiomyocytes^2:R1017, Madison, WI, USA) were stored in liquid nitrogen until thawed and cultured according to manufacturer instructions. Each well of the Agilent E-Plate CardioECR 48, E-Plate Cardio 96, or E-Plate Cardio View 96 (ACEA Biosciences, Inc., a part of Agilent Technologies, San Diego, CA, USA) was coated with 50 µL of a 1:100 diluted fibronectin (FN) solution at 10 µg/mL (F1114, Sigma-Aldrich, St. Louis, MO, USA) and incubated at 37 °C for at least one hour. This was followed by replacing fibronectin solution with 50 µL of prewarmed iCell cardiomyocyte-plating medium. Cells were thawed and diluted in prewarmed plating medium at 1,000,000 viable cells/mL. 50 µL of the cell suspension was transferred using a multichannel pipette, and seeded directly onto a precoated E-Plate CardioECR 48 (50,000 cells/well) in a laminar hood. The plates containing iCell cardiomyocytes^2 (iCell CM^{2}s) were kept in the hood at room temperature for 30 minutes, then placed and cultivated in a humidified incubator with 5% CO_2 at 37 °C. The plating medium was replaced with iCell cardiomyocyte maintenance medium for four hours postseeding. Medium change was performed every other day afterwards.

Electrical pacing of hiPSC-CMs using the ePacer

After cell seeding in the wells of the appropriate E-Plates, the E-Plates CardioECR 48, E-Plates Cardio 96, or E-Plates Cardio View 96 were placed in the Cardio or CardioECR cradles of ePacer and the impedance signals were recorded every four hours.

Following seven days of iCell CM^2 culture once the cells had established consistent and robust contractile activity as measured by impedance, electrical stimulation was continuously applied to the cells and progressively increased at fixed intervals. The cells were subjected to a pacing frequency starting at 1 Hz (for E-Plate Cardio 48, the stimulus setting was 0.85 V, 0.1 ms; for E-Plate Cardio 96, the stimulus setting was 1 V, 0.11 ms), followed by 1.5 Hz (for E-Plate CardioECR 48, the stimulus setting was 0.9 V, 0.12 ms; for E-Plate Cardio 96, the stimulus setting was 1.1 V, 0.12 ms) and ended at 2 Hz (for E-Plate CardioECR 48, 0.95 V, 0.18 ms; for E-Plate Cardio 96, the stimulus setting was 1.1 V, 0.15 V;). The duration of each applied pacing frequency was five days. The stimulus setting mainly depends on cell status and properties, which could vary. The entire pacing process takes approximately two weeks. Alternatively, a one-week maturation protocol can be used seven days postseeding. Cells are subjected to electrical stimulation at 1 Hz for one day, followed by a consecutive three-day pacing at 2 Hz using a similar stimulus setting to the two-week pacing protocol.

Chemical reagents

All the chemical reagents were purchased from Tocris (Minneapolis, MN, USA) and Sigma-Aldrich (St. Louis, MO, USA). 1,000-fold chemical stock solutions were prepared in DMSO and stored at −20 °C. The serial diluted chemicals (1,000-fold) were further prepared in DMSO immediately before compound addition. The 10-fold final dilution of the chemicals was prepared with culture medium for single use only. The final concentration of DMSO in the treated well was 0.1%.

The RTCA ePacer and assay principle

The xCELLigence RTCA ePacer is a dual-mode instrument that includes both real-time monitoring of hiPSC-CM viability and contraction as well as directed electrical pacing of hiPSC-CM. It consists of a workstation (computer and software), an electronic analyzer, a plate station that is placed inside a CO_2 incubator, and E-Plates, which are specialized electronic microplates (Figure 1B) placed within the cradles of the ePacer. Embedded within the bottom of the E-Plate wells are interdigitated gold microelectrodes, which noninvasively monitor cell impedance signal, providing both a measure of hiPSC-CM viability and, under high-frequency data acquisition mode, a measure of contractility (Figure 2B). The cellular impedance signal is recorded at a user-defined time interval (minutes and hours), and is reported using a unitless parameter called Cell Index. During the electrical pacing, the electrical pulses are directly applied to the cells through all the IMP electrodes on the bottom of the wells. For most cardiomyocytes, the length of each electrical pulse used by IMP electrode is less than 4 ms, which allows the contractile activities of cells to be immediately captured and recorded while the cells are being paced by IMP electrodes. The exact conditions for electrical pacing are dependent on the cell type, the inherent beating frequency, and the experimental context.

Three types of E-plates are compatible with the cradles of ePacer, including E-Plate CardioECR 48 (48-well plate), E-Plate Cardio 96, and E-Plate Cardio View 96 (96-well plate). The E-Plate Cardio View 96 contains a “view area” allowing imaging of the cells (Figure 1B).

The ePacer was developed and designed to be an integral component of a workflow that can be used in conjunction with both the xCELLigence Cardio and CardioECR systems as well as other orthogonal readouts such as calcium imaging, high-content imaging, fluorescent voltage dye imaging, and plate reader assays. Upon completion of the electrical pacing regimen on the ePacer, the plates containing the cells can either be transferred to xCELLigence Cardio or CardioECR instruments and continue to be monitored and treated with compounds. Alternatively, the cells within the plates can be processed appropriately and transferred to other types of instruments, as outlined in Figure 2C.

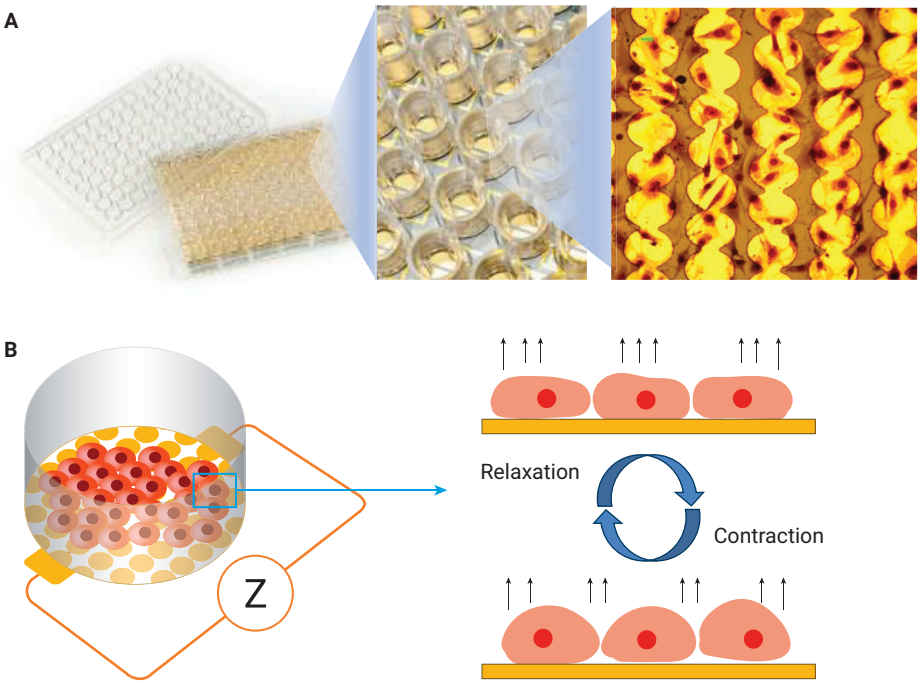


Figure 2. (A) An image of the Agilent xCELLigence E-Plate Cardio 96, which shows the interdigitated impedance (IMP) electrode array at the bottom of the wells. (B) Cardiomyocyte contraction and relaxation induce changes in cell morphology and cell attachment, which can be detected by impedance readout. (C) Full assay workflow using the Agilent xCELLigence RTCA ePacer. (1) Simply pace the cells on the ePacer, (2) assess maturity using the ePacer software, and (3) integrate into an assay platform of your choice.

Results and discussion

Using the ePacer for assessment of pacing efficiency and cell quality during chronic electrical stimulation

Figure 3A shows the dynamic changes in attachment and viability of iCell CM²s after seeding in E-Plate Cardio 96 for seven days, as measured by impedance and displayed as an overall Cell Index curve. Each data point in the overall Cell Index curve contains a recording of 15 seconds of high frequency impedance data, which represents the actual contraction of hiPSC-CMs generated at different time points along the cell culture. Real-time monitoring of both overall Cell Index and contraction in terms of rate and amplitude is a very useful parameter for quality assessment of hiPSC-CM. As the cells attach, they form a monolayer that is both physically and electrically coupled, culminating in synchronous and stable beating of the cells within seven days after cell seeding. To demonstrate the precision of directed electrical pacing, iCell CM²s were seeded in different wells and were subjected to electrical pacing at increasing frequencies. As shown in Figure 3B, the iCell CM²s were able to precisely follow the increasing frequency of the directed electrical pacing by displaying increased beating rate. Figure 3C shows a direct linear relationship between the pacing frequency and observation of increased beating rate.

With respect to directed electrical pacing, there are two important parameters to achieve optimal functional cells. These parameters include pacing efficiency as well as overall viability of hiPSC-CMs in response to directed electrical pacing. It is critical that the hiPSC-CMs follow the input pacing stimulus in a precise and uninterrupted manner. The ePacer automatically calculates and displays the number of wells that follow the input pacing

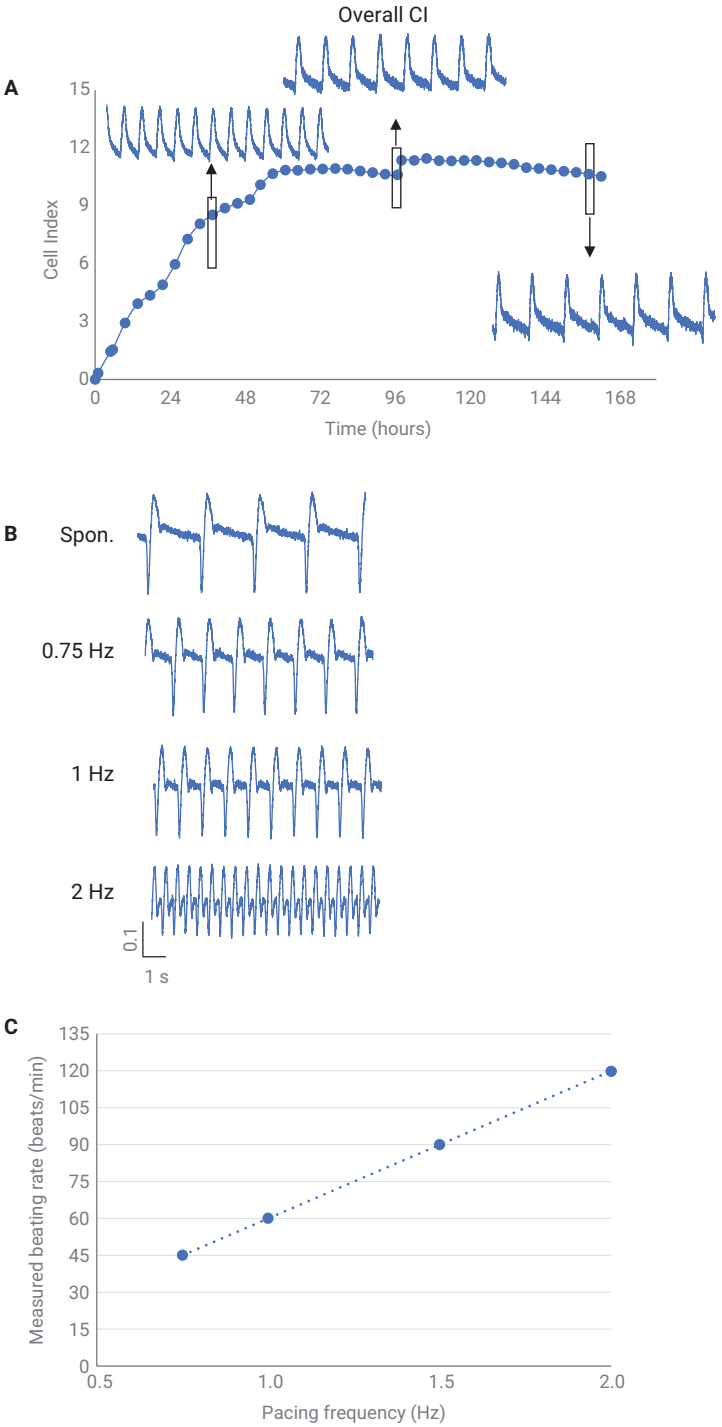


Figure 3. (A) The overall Cell Index curves were recorded in real time after cell seeding. Each data point in the Cell Index curve contains a recording of 15 seconds of IMP waveform data, which reflects the actual contraction of the cardiomyocytes, as shown in the figure. (B) These impedance waveforms show the cell beating rate response to the pacing workflow of the Agilent xCELLigence RTCA ePacer, from spontaneous beating to the 2 Hz target pacing frequency. (C) A linear relationship is observed between the pacing frequency and the cell beating rate. Continued next page.

stimulus (pacing efficiency, displayed as a percentage). This real-time display of pacing efficiency is important for making appropriate adjustments to input stimulus (Figure 3D). The data collected from two different test sites show high pacing efficiency in response to different input pacing stimuli (Table 1). This demonstrates that the ePacer is optimally designed to achieve consistent and reliable beating rates, which are important for functional maturation of hiPSC-CMs.

Cell quality and viability are other key factors affecting cell responses to the treatment. Typically, chronic electrical pacing should be performed under conditions that are not stressful to the cell and do not adversely impact cell viability. The ePacer monitors and displays overall cell health in real time both under pacing or nonpacing conditions. Figure 3E shows that there was no significant decrease in overall Cell Index obtained from paced cells compared to the nonpaced cells.

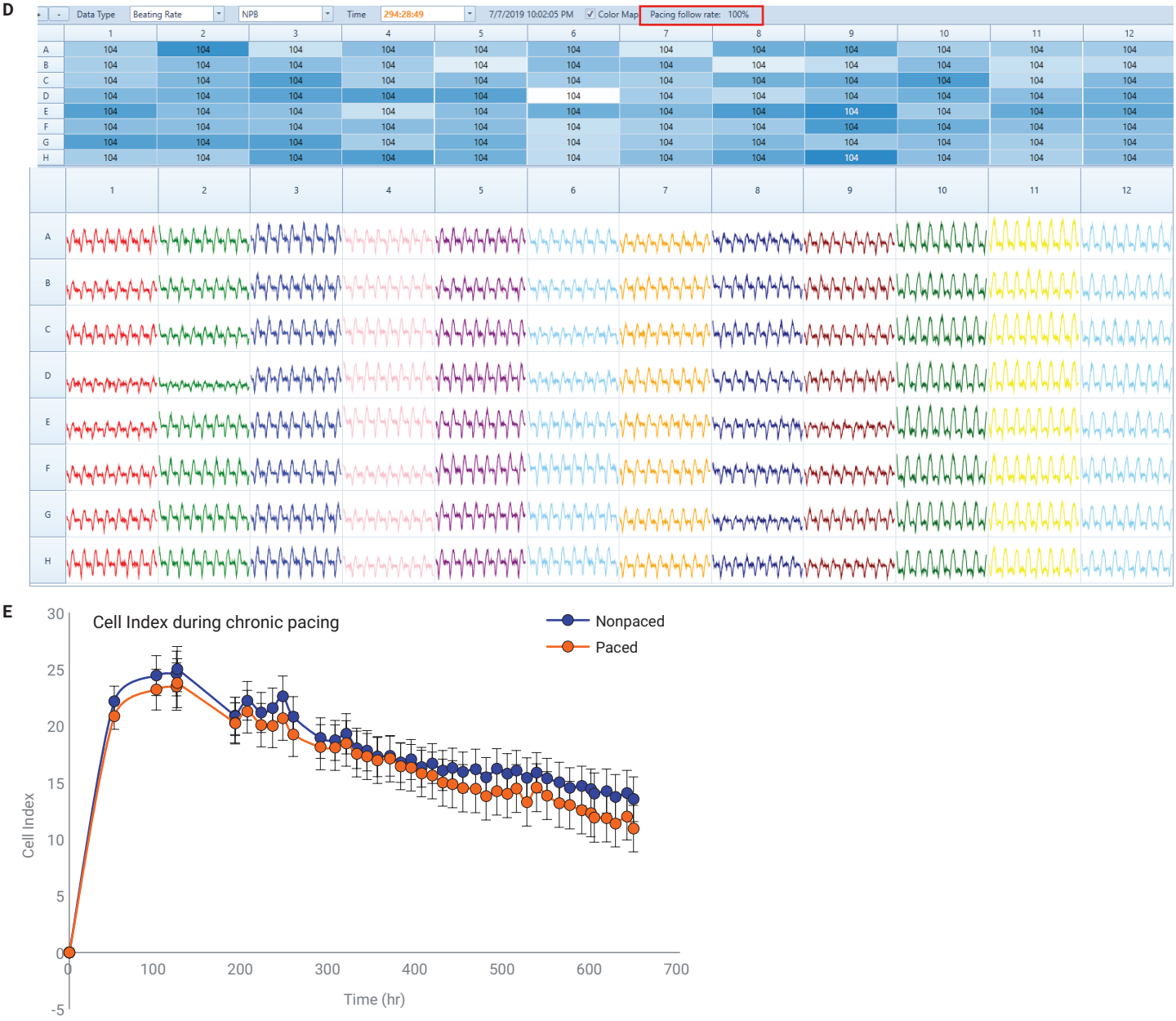


Figure 3. (D) The top section of this screenshot from the ePacer software shows the pacing efficiency of the cells when the electrical pacing is applied. The bottom section of the screenshot shows the cells contractile activity of each well in the plate. (E) The overall Cell Index was measured in nonpaced (CTRL) cells and electrically paced cells.

Table 1. Summary of the pacing capture efficiency of the Agilent xCELLigence RTCA ePacer during 2-week electrical pacing from two different test sites. The beating rate at different stages of the pacing process was presented as mean ±STDEV (n = 48 per site).

Beating Rate	Day 5_1Hz (60 beats)		Day 3/5_1.5 Hz (90 beats)		Day 5_2 Hz (120 beats)	
	Mean ±Std. Dev.	Pacing Efficiency	Mean ±Std. Dev	Pacing Efficiency	Mean ±Std. Dev	Pacing Efficiency
Site 1	59.8 ±0.7	100%	89.9 ±0.1	100%	118.1 ±3.7	90%
Site 2	59.9 ±0.12	100%	90.6 ±1.7	100%	121.4 ±1.9	100%

Directed electrical pacing reverses the BAmp and beating rate relationship and improves the functional response to inotropic compounds

One of the hallmarks of hiPSC-CM immaturity is the inherent negative force-frequency relationship.[18] To determine whether long-term electrical stimulation through the planar IMP electrodes impacts the functional maturation of hiPSC-CMs, we sought to determine the FF relationship in both nonpaced and electrically paced cells. Since measured impedance amplitude (BAmp) can be used as a surrogate for force of contraction, we evaluated the BAmp and beating rate relationship in the cells as a surrogate for the FF relationship. We cultured hiPSC-CMs in the E-Plates, and subjected half of the plate to directed electrical pacing (paced group) while maintaining the remaining half of the E-Plate without electrical stimulation (control group). The paced group was subjected to chronic electrical pacing, increasing the pacing frequency from 1 Hz (week 1) to 1.5 Hz (week 2) and finally 2 Hz (week 3) (Figure 4A). At the end of each pacing regimen, the hiPSC-CMs were subjected to acute electrical pacing with increasing frequencies (0.75, 1, 1.5, 2) as described previously. The data show that before applying stimulation to the cells after 1 week in culture (week 0), both paced and nonpaced cells displayed a negative BAmp-frequency relationship (Figure 4B and C). Continued stimulation at weeks 1 and 2 seemed to decrease the slope of the BAmp-frequency relationship, and at the end of week 3, a positive BAmp-frequency relationship was observed in the electrically paced group (Figure 4C). As a control, nonpaced cells were subjected to acute electrical stimulation, and continued to show negative BAmp-frequency relationship (Figure 4B). A shorter pacing protocol was also tested as described in the materials and methods section; it also showed a positive BAmp-frequency relationship (Figure 4D)

To determine if chronic electrical pacing of hiPSC-CM, as previously described, improved functional response of the cells to inotropic compounds, we first tested isoproterenol (ISO), a well known positive inotropic and chronotropic compound. As shown in Figure 5A, ISO (100 nM) treatment resulted in increased beating rate for both paced and nonpaced cells. However, the inotropic effect of ISO could only be observed in electrically paced cells, as demonstrated by an increase in the BAmp (52% ±14% SD) (shown in Figure 5B), while nonpaced cells showed a slight decrease in BAmp, as shown previously. We further expanded the functional test in paced cells by testing more positive inotropic compounds, with different mechanisms of action, as well as a negative inotropic compound. Table 2 shows that positive inotropic compounds profoundly increased beating amplitude only in paced cells and not in nonpaced cells (data not shown). Conversely, a negative inotropic compound, isradipine, decreased beating amplitude in paced and nonpaced cells. In summary, chronic electrical pacing converts hiPSC-CM response from an inherent negative BAmp-frequency relationship to a positive BAmp-frequency relationship and improves the cell contractile responses to positive inotropes, suggesting that long-term electrical pacing improves the maturation status of hiPSC-CMs at the functional level.

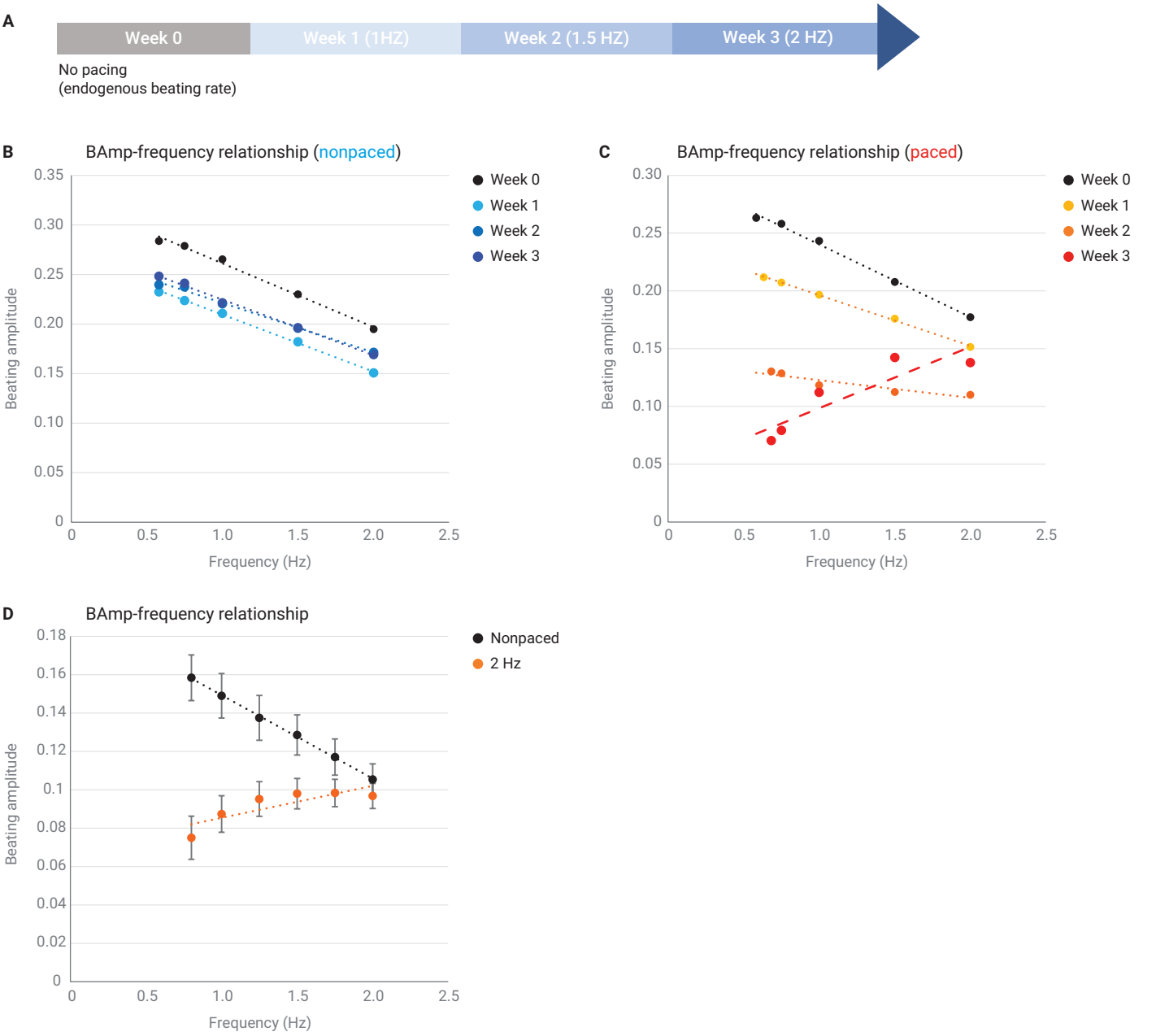
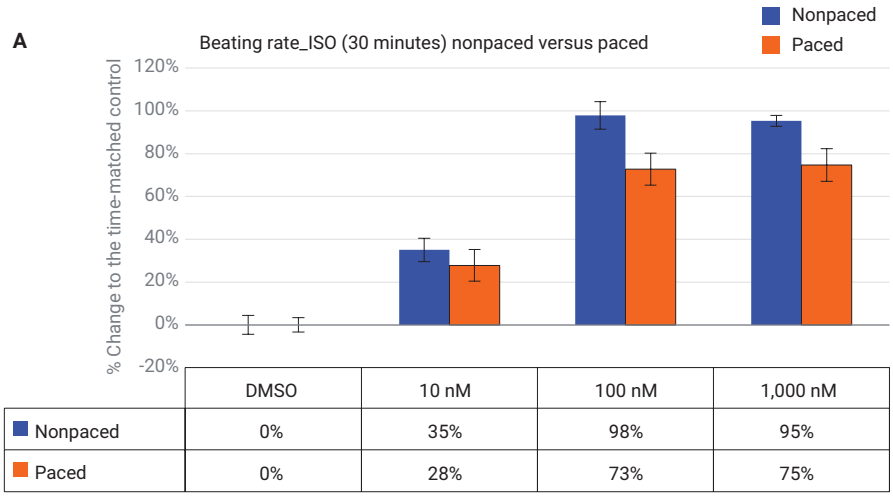


Figure 4. The timeline of chronic electrical pacing protocol (A). Beating amplitude (BAmp) and beating rate relationship was obtained from (B) nonpaced cells and (C) paced cells in week 0, the time before chronical pacing started; week 1, the time before 1 Hz pacing ended; week 2, the time before 1.5 Hz pacing ended; and week 3, the time before 2 Hz pacing ended.

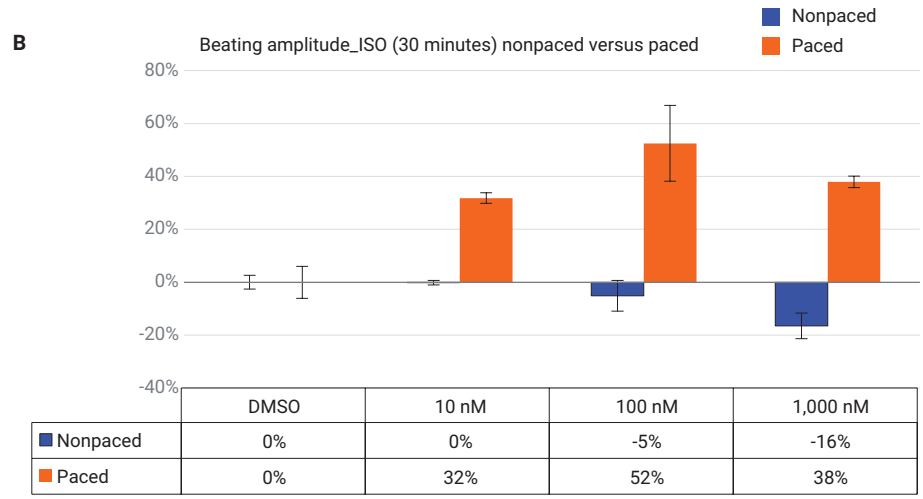
Conclusion

hiPSC-CMs are derived from reprogrammed somatic cells. Numerous lines of evidence, including morphological, structural, gene and protein expression, and functional, indicate that these cells display the hallmarks of fetal rather than adult cardiomyocytes 29, and various *in vitro* approaches have failed to recapitulate the natural cardiomyocytes development program. One of the key functional consequences of the inherent immaturity and lack of sophisticated myocyte structure of hiSPC-CM is generation of a negative force-frequency (NFF) relationship.[18,19] Using IMP measurement as a surrogate for contractility and directed electrical pacing to control the rate of contraction, our results demonstrate that hiPSC-CMs display a negative BAmp-frequency relationship (Figure 4A), confirming previous observations that hiPSC-CM display a NFF relationship.

To improve the contractile maturation of hiPSC-CMs, we developed a novel instrument, the Agilent xCELLigence RTCA ePacer, which uses microplates embedded with electrodes to perform directed electrical pacing of the cells in a highly controlled and consistent fashion for both acute and chronic stimulation. In this study, we used the ePacer to perform chronic electrical pacing of hiPSC-CMs, ranging from 1 to 2 Hz. Our results confirm the earlier findings that electrical pacing of hiPSC-CM can lead to further maturation.[27,30] In addition, our data clearly demonstrate for the first time that the conditions we used for electrical pacing reverse the negative BAmp-frequency relationship of hiPSC-CM, and result in a positive BAmp-frequency relationship, indicating that electrical stimulation can lead to further functional maturation of hiPSC-CM (Figure 4). Furthermore, the mature FF relationship observed in chronically paced cells allows the cells to respond to positive inotropic compounds in an appropriate and dose-dependent manner (Figure 5B and Table 2).



	DMSO	10 nM	100 nM	1,000 nM
Nonpaced	0%	35%	98%	95%
Paced	0%	28%	73%	75%



	DMSO	10 nM	100 nM	1,000 nM
Nonpaced	0%	0%	-5%	-16%
Paced	0%	32%	52%	38%

Figure 5. Isoproterenol, a known positive inotrope, was added to both nonpaced and paced cells. (A) The % change in beating rate 30 minutes after isoproterenol (ISO) addition to nonpaced beating cells (blue bar) and chronically paced cells (orange bar) at different concentrations. (B) The % change in BAmp obtained from nonpaced beating cells (blue bar) and chronically paced cells (orange bar). The data were represented by mean ±STDEV (n ≥3).

In addition to the powerful electrical pacing function, the ePacer system comes equipped with its own software, allowing both real-time monitoring of the cells in terms of viability and contractility, and allowing precise programming of all the pacing parameters in terms of magnitude, duration, and shape of the input stimulus. It also allows defined and controlled pacing protocols while providing the throughput and flexibility needed to screen compounds or multiple replicates. The automatic FF relationship evaluation feature of the ePacer software helps the user to determine when the paced cells have developed a positive force frequency relationship, which is an attribute of matured cardiomyocytes.

The compatibility of the E-Plate Cardio View 96 with orthogonal readouts such as calcium and high-content imaging allows integration of this system into various workflows depending on the experimental needs.

Table 2. Summary of contractile responses of chronic electrically paced iCell CM^{2}s. After long-term pacing, the cells were exposed to both positive and negative inotropic compounds. The % change of BAmp after compound addition was further calibrated to the time-matched DMSO control wells. The data were represented by mean ±STDEV (n ≥3).

	Bay K8644 (67 nM)	**Pimobendan (10 µM)**	**Milrinone (30 µM)**	**Omecamtiv mecarbil (600 nM)**	**Digoxin (100 nM)**	**Isradipine (10 nM)**
MOA	L-type Ca^{2+} ion channel activator	Ca^{2+} sensitizer	Phosphodiesterase-3 inhibitor	Myosin activator	Na-K ATP exchanger inhibitor	L-type Ca^{2+} ion channel inhibitor
Inotropic Effect	Positive	Positive	Positive	Positive	Positive	Negative
% Change (BAmp)	32 ±5%	31 ±6%	30 ±12%	58 ±15%	175 ±35%	-47 ±7%

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