Automaticity and conduction properties of bio-artificial pacemakers assessed in an \textit{in vitro} monolayer model of neonatal rat ventricular myocytes

Yau-Chi Chan¹, Hung-Fat Tse¹,²*, Chung-Wah Siu¹,², Kai Wang¹, and Ronald A. Li¹,²,³*

¹Cardiology Division, Department of Medicine, University of Hong Kong, Queen Mary Hospital, Hong Kong, SAR; ²Stem Cell and Regenerative Medicine Programme, Research Centre of Heart, Brain, Hormone and Healthy Ageing, University of Hong Kong, Hong Kong, SAR; and ³Center of Cardiovascular Research, Mount Sinai School of Medicine, New York, NY, USA

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Aims
A better understanding of the ionic mechanisms for cardiac automaticity can lead to better strategies for engineering bio-artificial pacemakers. Here, we attempted to better define the relative contribution of $I_f$ and $I_{K1}$ in the generation of spontaneous action potentials (SAPs) in cardiomyocytes (CMs).

Methods and results
Monolayers of neonatal rat ventricular myocytes (NRVMs) were transduced with a recombinant adenovirus (Ad) to express a gating-engineered HCN1 construct (HCN1-ΔΔΔ) for patch-clamp and multielectrode array (MEA) recordings. Single NRVMs exhibited a bi-phasic response in the generation of SAPs (62.6 ± 17.4 b.p.m., Days 1–2; 194.3 ± 12.3 b.p.m., Days 3–4; 73% quiescent, Days 9–10). Although automaticity time-dependently decreased and subsequently ceased, $I_f$ remained fairly stable ($25.2 \pm 1.1$ pA/pF, Days 1–2; $25.1 \pm 1.4$ pA/pF, Days 7–8; $24.3 \pm 1.3$ pA/pF, Days 13–14). In contrast, $I_{K1}$ declined rapidly (from $216.9 \pm 2.7$ pA/pF on Days 1–2 to $24.4 \pm 1.6$ pA/pF on Days 5–6). Maximum diastolic potential/resting membrane potential ($r = 0.89$) and action potential duration at 50% (APD$_{50}$, $r = 0.73$) and 90% (APD$_{90}$, $r = 0.75$) but not the firing rate ($r = -0.3$) were positively correlated to the $I_{K1}$. Similarly, monolayer NRVMs ceased to spontaneously fire after long-term culture. Ad-HCN1-ΔΔΔ transduction restored pacing in silenced individual and monolayer NRVMs but with reduced conduction velocity and field potential amplitude.

Conclusion
We conclude that the combination of $I_{K1}$ and $I_f$ primes CMs for bio-artificial pacing by determining the threshold. However, $I_f$ functions as a membrane potential oscillator to determine the basal firing frequency. Future engineering of automaticity in the multicellular setting needs to have conduction taken into consideration.

Keywords
$I_f$ • $I_{K1}$ • HCN • Automaticity • Action potential

Introduction
Neonatal rat ventricular myocytes (NRVMs) have been commonly employed as an \textit{in vitro} model for studying cellular electrophysiological properties of the heart. When cultured as a confluent monolayer, NRVMs beat spontaneously up to 40 days.¹,² The pacemaker current ($I_f$), encoded by hyperpolarization-activated cyclic nucleotide-modulated (HCN) channel gene family, is known to play an important role in mediating automaticity. $I_f$, activated upon hyperpolarization and modulated by intracellular cAMP, depolarizes cells to the action potential (AP) threshold (a.k.a. take-off potential or TOP). We previously demonstrated that focal transduction of an engineered HCN channel in a swine model of sick-sinus syndrome suffices to generate a stable in vivo bio-artificial sinoatrial (SA) node for physiological pacing.³ Overexpression of HCN2 in NRVMs likewise leads to robust $I_f$ expression and promotes automaticity,⁴ although the underlying ionic basis has not been fully explored. In contrast, dominant-negative
suppression of HCN2 channels markedly reduces native \( I_f \) and even silences spontaneous firing of NRVMs.\(^5\) Despite the various lines of functional evidence, the mechanistic role of \( I_f \) has been questioned due to its intrinsically slow kinetics and negative activation relative to the time scale and voltage range of cardiac pacing.\(^6\)–\(^8\) On the basis of the experimental observation that \( I_{K1} \) suppresses unloading of the latent pacemaker activity of normally silent adult ventricular cardiomyocytes (CMs), it has been suggested that down-regulation of \( I_{K1} \), rather than the up-regulation of \( I_f \), is the key to automaticity.\(^9\),\(^10\) Developmentally, \( I_{K1} \) densities markedly increase in the foetal and neonatal hearts to shorten the action potential duration (APD) and hyperpolarize the resting membrane potential (RMP).\(^11\)–\(^15\) Kir2.1 overexpression increases the conduction velocity (CV) by improving the availability of voltage-gated Na\(^+\) channels for opening.\(^16\) However, the functional interaction between \( I_{K1} \) and \( I_f \) is just beginning to be understood. In a series of computational and functional studies, we demonstrated that the relative \( I_f/I_{K1} \) activity is crucial for both the induction and the modulation of pacemaking.\(^17\)–\(^20\) Using NRVMs as an in vitro model, here, we tested the hypothesis that alterations of the balance of \( I_f \) and \( I_{K1} \) activities directly lead to changes in the generation of spontaneous action potentials (SAPs). The results are discussed in the context of the dissected individual functional roles of \( I_f \) and \( I_{K1} \) in cardiac automaticity.

**Methods**

In this study, the time-dependent changes of SAP generation as well as the relationship of \( I_f \) and \( I_{K1} \) were examined using a combination of adenovirus (Ad)-mediated gene transfer and electrophysiological techniques.

**Cardiomyocyte isolation and culture**

Ventricles from neonatal Wistar rats (0- to 1-day-old) sacrificed by decapitation were quickly removed, rinsed four times with modified Hank’s solution, and minced into small pieces on ice. The tissue fragments were then transferred into a 50 mL Falcon tube with the addition of 10 mL of 0.2% trypsin pre-warmed to 37°C. The tube was placed in a water bath on the top of a hot plate stirrer stirring the tissue fragments with a magnetic bar for 10 min at 37°C. The supernatant was discarded to remove dead and blood cells. After that, fresh pre-warmed 0.2% trypsin was added to digest the minced myocardium for another 5 min at 37°C. To stop the digestion, the supernatant was aspirated gently, and the cells were re-suspended in NRVM culture medium were pre-plated for 1 h. Again, the supernatant was aspirated, and the cells were plated in six-well plates or MEA dishes at the density of \( 6 \times 10^5 \) cells/mL. Culture media were changed every day. These procedures reproducibly generate monolayer cultures that contract synchronously at \( >300 \) b.p.m. consistent with the normal beating rate of the intact rat heart. For patch-clamping, monolayer cultures were re-suspended by brief exposure to trypsin–EDTA. The cells were re-plated onto gelatin-coated cover slips at a lower density (\( 2 \times 10^5 \) cells/mL), allowing the cells to settle down overnight subjecting to study within 14–24 h.

**Gene transfer**

Adenovirus-mediated HCN1 gene transfer was performed as we described previously.\(^17\),\(^19\) In brief, the bicistronic Ad shuttle vector pAdCMV-GFP-IREs (pAdCGI) was employed. Internal ribosomal entry site (IRES) allows the simultaneous translation of two transgenes with a single transcript and, in our experiments, GFP and an HCN1 construct. HCN1Δ235–237-GFP (HCN1-ΔΔΔ) with shortened S3–S4 linker was cloned into the second position of pAdCGI at EcoRI and XmaI to generate pAdCGI-HCN1-ΔΔΔ (pAdHCN1-ΔΔΔ).\(^21\) Adenoviruses were generated by Cre-lox recombination of purified \( \psi \) 5 viral DNA and shuttle vector DNA using Cre+ cells. The recombinant products were plaque purified, amplified, and purified again by CsCl gradients, yielding concentrations on the order of \( 10^{10} \) plaque-forming units per 1 mL. As needed, NRVMs were transduced with Ad-CGI or Ad-HCN1-ΔΔΔ at \( \sim2 \times 10^5 \). A transduction efficiency of \( \sim70–80\% \) could be typically achieved. The dishes were kept at 37°C in a humidified atmosphere of 95% O\(_2\) and 5% CO\(_2\) overnight, and then the supernatant was discarded. The dishes were washed, refilled with the normal culture medium, and remained in an incubator for 1 day before electrophysiological experiments.

**Electrophysiological recording**

In patch-clamp experiments, ionic current recordings were performed in the whole-cell mode with individual cells isolated from monolayers being superfused at room temperature (\( \sim23°C \)). For AP recordings at 36 ± 0.5°C, the perforated-patch technique was employed with 100 \( \mu \)M amphotericin B added to the pipette solution.\(^22\) The electrode tip resistances in both cases were \( \sim3–4 \) MΩ, whereas the sampling frequencies were 1.25 and 2.00 kHz, respectively. For \( I_f \) recordings, external [K\(^+\)]\(_o\) was increased to 25 mM. BaCl\(_2\) 2 mM, CdCl\(_2\) 200 \( \mu \)M, and 4-aminopyridine 4 mM were added to block \( I_{K1} \), \( I_{Ca-L} \), and \( I_{Na} \), respectively. \( I_f \) was measured as the difference between the instantaneous current at the beginning of a hyperpolarizing step, ranging from \( -30 \) to \( -140 \) mV in 10 mV increments, and the steady-state current at the end of the 3 s hyperpolarizing step. For \( I_{K1} \) recordings, external [K\(^+\)]\(_o\) was also increased to 25 mM. Two hundred micromolars of CdCl\(_2\) were added to block \( I_{Ca-L} \), \( I_{Na} \) was inactivated by a holding potential of \( -30 \) mV. For Ba\(^2+\)-sensitive \( I_{K1} \), currents recorded before and after the addition of 2 mM BaCl\(_2\) were subtracted. Since measurements of ionic current and AP involved different pipette solutions, cells were subjected to either recording at a time. Averages of 6–16 measurements were performed for each set of parameters.

**Multielectrode array recording**

*In vitro* multielectrode array (MEA) recordings were performed at 37°C by simultaneously measuring extracellular field potentials from 60 microelectrodes (of 30 \( \mu \)m diameter) arranged in an 8 × 8 layout grid with a 200 \( \mu \)m inter-electrode distance. Six monolayers of NRVMs were cultured on gelatin-coated MEA plates for experiments. The raw signals were collected at 25 kHz, bandpass filtered, and amplified (Multi-channel Systems), followed by analysis with MC Data Tool V1.3.0 to generate a conduction map based on the time differences at which signals were detected at each of the microelectrodes. Filtered signals were differentiated digitally to determine the local activation time (LAT) at each electrode. Colour-coded activation maps were constructed by plotting the LAT values against the electrode sites. Activation maps were generated using the Matlab standard two-dimensional plotting function (pcolor) (Matlab 5.3; Mathworks Inc.). Conduction velocity was calculated using the first method described previously by Meiry et al.\(^23\)

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Statistical analysis

All data reported are means ± SEM. Statistical significance was determined for all individual data points and fitting parameters using one-way ANOVA with Tukey’s HSD post hoc test. Furthermore, correlation analysis was performed to examine the relationships between various AP parameters and $I_{K1}$ properties. Calculations were performed with OriginPro 7.5 software (OriginLab Corporation, Northampton, MA, USA). A $P$-value of < 0.05 was considered statistically significant.

Results

Bi-phasic changes of the spontaneous action potentials of individual neonatal rat ventricular myocytes

Representative AP tracings recorded from individual NRVMs at different time points are given in Figure 1. Same as previous studies,11,14 no SAPs were observed from freshly isolated NRVMs ($n = 12$). After 24 h culturing, SAP firing ($n = 5$) with a mean frequency of $62.6 ± 17.4$ b.p.m., a relatively hyperpolarized maximum diastolic potential ($MDP = −71.0 ± 1.8$ mV), rapid AP upstroke ($288.2 ± 41.6$ mV/s), and Phase 4 depolarization ($43.6 ± 12.4$ mV/s) could be recorded. Action potential duration at 50% ($APD_{50}$) and $APD_{90}$ were $81.4 ± 10.5$ and $144.4 ± 7.6$ ms, respectively (Figure 2). On Days 3–4 in culture, AP firing significantly increased and peaked at $194.3 ± 12.3$ b.p.m. ($n = 10$) ($P < 0.05$). A gradual time-dependent decrease was observed until SAPs completely disappeared on Days 13–14 ($n = 12$). Maximum diastolic potential further depolarized and stabilized at $−63.0 ± 2.5$ mV on Days 5–6. In Figure 2C, on Days 1–2 and 3–4 of culture, $APD_{90}$ was relatively short at $144.4 ± 7.6$ and $141.1 ± 5.7$ ms, respectively. It then prolonged ($212.1 ± 1.2$ ms on Days 5–6 and $198.5 ± 18.0$ ms on Days 7–8, $P < 0.05$) when automaticity began to slow down and the recovered cells silenced from Days 9 to 14 ($170.2 ± 31.4$ ms on Days 13–14). In contrast to the bi-phasic change, both the maximum upstroke velocity and Phase 4 depolarization slope continued to decrease with the TOP becoming more and more depolarized during the course of our experiments (Figure 2D–F).

$I_f$ is not responsible for the time-dependent loss of automaticity, but its overexpression suffices to induce pacing in otherwise silenced neonatal rat ventricular myocytes

$I_f$ was elicited by hyperpolarizing steps to $−140$ mV from a holding potential of $−30$ mV. Under the physiological extracellular $K^+$ concentration ($[K^+]_o$) of $5$ mM at room temperature, no measurable hyperpolarization-activated inward current could be recorded from NRVMs (data not shown). As reported previously,5 $I_f$ was determined after increasing $[K^+]_o$ to $25$ mM (Figure 3A). Interestingly, although the automaticity time-dependently decreased and subsequently became abolished in culture, $I_f$ remained fairly stable ($−5.2 ± 1.1$, $−5.1 ± 1.4$, and $−4.3 ± 1.3$ pA/pF on Days 1–2, 7–8, and 13–14, respectively; $P > 0.05$) (Figure 3B).

Furthermore, the steady-state activation curves on Days 1 and 13 were identical; the half activation potentials ($V_{1/2}$) were $−109.1 ± 4.2$ and $−113.2 ± 1.7$ mV, respectively ($P > 0.05$; Figure 3C).

Transduction of NRVMs with Ad-HCN1-ΔΔΔ on Day 13 led to robust expression of $I_{K1}$ with a current density of $−27.5 ± 4.7$ pA/pF at $−140$ mV under the physiological $[K^+]_o$. Yet, it was significantly larger than that of control cells recorded at $[K^+]_o$ of $25$ mM (Figure 3A and B). The activation curve also became significantly shifted in the positive direction with a $V_{1/2}$ of $−56.3 ± 4.5$ mV (Figure 3C), consistent with what we previously reported for the gating-engineered construct that opens more readily than the wild-type (WT) counterparts.21 The firing rate of 13-day-old quiescent cells 1 day after transduction was $104.8 ± 18.9$ b.p.m. (Figures 1 and 2A). In addition, depolarization of MDP ($−52.3 ± 3.8$ mV) and prolongation of APD ($APD_{50} = 572.6 ± 70.2$ ms; $APD_{90} = 707.0 ± 80.2$ ms) were also observed (Figure 2B and C).

Correlation between $I_{K1}$ and automaticity

At $−120$ mV, the $I_{K1}$ density of was $−16.9 ± 2.7$ pA/pF on Days 1–2. This declined rapidly to $−4.4 ± 1.6$ pA/pF on Days 5–6, then modestly recovered to about $−6.0$ pA/pF by the end of 14 days (Figure 4B). The current–voltage relationships of $I_{K1}$ in culture are shown in Figure 4C. The reversal potential remained unchanged. Since $I_{K1}$ displayed a bi-phasic change similar to that of SAP (cf. Figure 2A), correlation analysis was performed to examine the relationships between various AP parameters and $I_{K1}$ properties. The MDP/RMP ($r = 0.89$), $APD_{50}$ ($r = 0.73$), and $APD_{90}$ ($r = 0.75$) of NRVMs during culture were positively correlated to the $I_{K1}$ current density at $−120$ mV (Figure 5A and B). In contrast, no obvious correlations were found between firing rate and $I_{K1}$ density ($r = −0.3$; Figure 5C). The relative $I_{f}/I_{K1}$ ratio at $−120$ mV on individual NRVMs during culture showed a sudden increase on Days 3–4 ($0.88 ± 0.40$) to the maximum value on Days 5–6 ($2.25 ± 0.54$; $P < 0.05$). Thereafter, it gradually decreased to $−56$% of the maximum on Days 13–14 ($1.27 ± 0.62$) (Figure 5D).

The automaticity change of monolayer culture of neonatal rat ventricular myocytes

Unlike individual NRVMs (Figure 2A), synchronized spontaneous firing of monolayers disappeared as soon as after 3 days in culture. The initial firing rate of the monolayer was $36 ± 7.9$ b.p.m., about twice slower than that of individual NRVMs on Day 1 as gauged by single-cell current-clamp recordings. The time to the peak firing rate was also delayed (on Day 8) compared with single cells (on around Day 4) (Figures 2A and 6A). At its fastest (i.e. Day 8), however, the firing rate of monolayers was comparable ($220 ± 29.5$ b.p.m.). This slowly declined to $98 ± 25.6$ b.p.m. on Day 40 and became completely ceased on Day 48 (Figures 6A and 7). Transduction with Ad-HCN1-ΔΔΔ led to spontaneous beating with a firing rate of $86.0 ± 23.7$ b.p.m.

Along with the decrease in spontaneous firing, both the CV and field potential amplitude of the monolayer also time-dependently reduced to about half from $75.3 ± 9.4$ μm/ms and $9.43 ± 2.02$ mV on Days 1–2 and 13–14, respectively (Figures 6B and C).

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Figure 1 Representative SAPs recorded from control and HCN1-ΔΔΔ-transduced NRVMs at 36°C. After 14 days of culture, SAP was abolished but a single AP could be elicited upon electrical stimulation of 0.1–1 nA for 5 ms. Spontaneous action potentials restored 1 day after HCN1-ΔΔΔ transduction. Single APs were magnified to the right for comparison.
569.3 ± 39.2 μV on Day 8 to 36.0 ± 3.4 μV/ms and 301.4 ± 30.8 μV on Day 43, respectively (Figure 6B and C). The CV reduction was irreversible even after Ad-HCN1-DDD transduction. Noticeably, after Ad-HCN1-DDD transduction, the field potential amplitude reduced further and significantly to 57.9 ± 5.5 μV, about one-sixth of that on Day 43 (Figure 6C; P < 0.05). Colour-coded activation maps showed that the activation (firing) origin changed during culture (Figure 8).
Discussion

In the present study, we investigated cardiac automaticity in individual cells and monolayers of NRVMs. Our key findings are as follows. For individual cells, (1) spontaneous AP displays a bi-phasic change in the firing rate with a peak appearing early on Days 3–4; (2) lack of changes of \( I_f \) and \( I_{K1} \) down-regulation cannot account for the time-dependent loss of automaticity change; (3) automaticity can be conferred on silenced single cells or monolayer upon gene transfer of HCN1-\( \Delta \Delta \). As for monolayers, (4) spontaneous AP likewise displays a bi-phasic change in the firing rate but this automaticity sustains for about a month longer than individual cells with comparable maximal firing rates; (5) AP firing rate, CV, and field potential similarly exhibit a trend of gradual decrease in magnitude over time; (6) and gene transfer of HCN1-\( \Delta \Delta \) in quiescent cultures partially restores the spontaneous beating.

Several gene-based approaches have been explored to confer upon non-pacing CMs the ability to intrinsically fire APs like genuine nodal pacemaker cells. Protein engineering has been recently applied as an alternative to overexpress WT HCN1 or 2 channels alone in normally quiescent ventricular CMs and cause cellular rhythmic oscillations.\(^4,17\) Mathematically, AP was simulated using a computational model to understand the contribution of \( I_f \) to proper pacing.\(^18\) Pacemakers were then engineered into non-pacing CMs via somatic gene transfer of HCN1-\( \Delta \Delta \) both in vitro and in vivo.\(^21\) In a sick-sinus syndrome porcine model, physiological heart rhythms can be restored via focal transduction of the left atrium via Ad-HCN1-\( \Delta \Delta \) injection (as opposed to the right atrium where the native SA node is anatomically located). Mechanistically, we have reported that a fine balance between \( I_f \) and \( I_{K1} \) is key to successful automaticity induction in both atrial and
ventricular CMs. In NRVMs, Qu et al. and Er et al. have demonstrated its critical role in SAP generation and pacing modulation. In the present study, no measurable If could be recorded at physiological $[K^+]_o$ (5 mM). Furthermore, its current density at $[K^+]_o$ of 25 mM and activation relation remained unchanged during the time course of our experiments, in sharp contrast to the bi-phasic change of SAP. This finding suggested that If alone is not responsible for the time-dependent loss of automaticity of NRVMs in culture. However, this could be rescued by forced expression of If via Ad-mediated gene transfer in both silenced individual and monolayer NRVMs, although the firing rates were slower.

**Figure 5** (A) The MDPs or RMPs of NRVMs were plotted against $I_{K1}$ at the same time point. A strong linear correlation ($r = 0.89$) between the bi-phasic change of MDP (or RMP) and $I_{K1}$. (B) The correlation between APD$_{50}$ and $I_{K1}$ is relatively weak ($r = 0.73$ and 0.75, respectively). (C) Correlation was absent between firing rate and $I_{K1}$ current density. (D) Change of $I_{K}$ to $I_{K1}$ ratio ($\|I_{K}/I_{K1}\|$) at $-120$ mV on NRVMs during culture ($n = 16$), $*P < 0.05$.

**Figure 6** Summary of MEA recording parameters of NRVM monolayer. (A) Firing rate of control and Ad-HCN1-$\Delta\Delta\Delta$-transduced monolayer cultures. (B) Conduction velocity. (C) Amplitude of field potential ($n = 6$), $*P < 0.05$. 

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Compared with $I_f$, the relationships between $I_{K1}$ and AP have been much better defined. In addition to its contribution to Phase 3 repolarization, $I_{K1}$ stabilizes a negative RMP ($-80$ mV) and suppresses any latent spontaneous electrical activity. This was demonstrated by Hirano et al.\textsuperscript{125} and Imoto et al.\textsuperscript{126} in 1988 that blockade of $I_{K1}$ by Ba\textsuperscript{2+} induces automaticity in isolated guinea pig ventricular myocytes. Miake et al.\textsuperscript{10} confirmed these findings using a genetic approach to dominant-negatively suppress Kir2-encoded $I_{K1}$ and thereby unleash the latent pacemaker activity of normally silent ventricular myocytes. On the basis of these findings, the absence of the strongly polarizing $I_{K1}$, rather than the presence of $I_f$, has been proposed as a key factor for pacing.\textsuperscript{9} As presented in this study, $I_{K1}$ time-dependently down-regulates in a manner reciprocal to the slowing of the firing rates, although $I_f$ remained essentially unchanged. These results are in complete accordance with the view that effective induction and modulation of cardiac automaticity involves components other than $I_{K1}$.

In this study, the automaticity of individual NRVMs in culture could only maintain up to 2 weeks. However, that of monolayer culture recorded by MEA lasted up to 48 days. Such differences in the electrical properties of the monolayer culture from those of single cells can be attributed to cell–cell coupling. Colour-coded activation maps indicate that one or multiple pacemaking sites can arise during long-term culture (Figure 8). Of note, the

Figure 7 Representative MEA recordings. Automaticity remained up to 48 days and could be rescued by Ad-HCN1-ΔΔΔ transduction.
presence of a few firing cells may suffice to lead to firing of the entire monolayer via electrical coupling through gap junctions. Consistently, Yasui et al.\textsuperscript{27} have demonstrated that gap junction channels are important for maintaining the electrophysiological activities of cultured NRVMs. Ad-HCN1-ΔΔΔ-induced automaticity in silenced NRVMs is associated with a reduction of CV. Some possible underlying mechanisms include down-regulation of connexin-mediated gap junction for cell–cell coupling, alterations of voltage-gated Na\textsuperscript{+} channel for rapid conduction of APs, etc. Taken together, our results implicate that further development of bio-artificial pacemakers should have conduction properties taken into consideration, an area that has not been extensively explored.

In summary, we have employed long-term culturing of NRVMs as an in vitro model for investigating the role of $I_{K_1}$ and $I_f$ in cardiac automaticity at the single- and multicellular levels. On the basis of the present and other results, we conclude that while the combination of $I_{K_1}$ and $I_f$ primes CMs for bio-artificial pacing (by determining the threshold), $I_f$ functions as a membrane potential oscillator to determine the basal firing frequency. For multicellular preparations, the engineering of automaticity needs to have conduction taken into consideration.

**Figure 8** Colour-coded activation maps from a representative culture showing the pacing origin and the decrease in CV. The scales of the maps vary from 0 to 65 ms.
Conflict of interest: none declared.

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