Rapid ionic current phenotyping (RICP) identifies mechanistic underpinnings of iPSC-CM AP heterogeneity

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Abstract

As a renewable, easily accessible, human-derived in vitro model, human induced pluripotent stem cell derived cardiomyocytes (iPSC-CMs) are a promising tool for studying arrhythmia-related factors, including cardiotoxicity and congenital proarrhythmia risks. An oft-mentioned limitation of iPSC-CMs is the abundant cell-to-cell variability in recordings of their electrical activity. Here, we develop a new method, rapid ionic current phenotyping (RICP), that utilizes a short (10 s) voltage clamp protocol to quantify cell-to-cell heterogeneity in key ionic currents. We correlate these ionic current dynamics to action potential recordings from the same cells and produce mechanistic insights into cellular heterogeneity. We present evidence that the L-type calcium current is the main determinant of upstroke velocity, rapid delayed rectifier K⁺ current is the main determinant of the maximal diastolic potential, and an outward current in the excitable range of slow delayed rectifier K⁺ is the main determinant of action potential duration. We measure an unidentified outward current in several cells at 6 mV that is not recapitulated by iPSC-CM mathematical models but contributes to determining action potential duration. In this way, our study both quantifies cell-to-cell variability in membrane potential and ionic currents, and demonstrates how the ionic current variability gives rise to action potential heterogeneity. Based on these results, we argue that iPSC-CM heterogeneity should not be viewed simply as a problem to be solved but as a model system to understand the mechanistic underpinnings of cellular variability.

New & Noteworthy: We present rapid ionic current phenotyping (RICP), a current quantification approach based on an optimized voltage clamp protocol. The method captures a rich snapshot of ionic currents that
provides quantitative information about multiple currents (e.g., $I_{CaL}$, $I_{Kr}$) in the same cell. The protocol helped to identify key ionic determinants of cellular action potential heterogeneity in iPSC-CMs. This included unexpected results, such as the critical role of $I_{Kr}$ in establishing the maximum diastolic potential.

**Running Head:** Rapid ionic current phenotyping of iPSC-CMs

**Keywords:** Induced pluripotent stem cells, Patch clamp, Arrhythmias, Ion channels, Computer simulation
1 Introduction

Human induced pluripotent stem cell derived cardiomyocytes (iPSC-CMs) are a promising model to study congenital (Terrenoire et al., 2013; Han et al., 2014) and acquired (Mathur et al., 2015) cardiac arrhythmias. iPSC-CMs can be derived from patient cells taken through a minimally invasive blood draw or skin biopsy. The resultant cells contain the genetic information of donors, and offer the potential to study patient-specific phenotypes in the lab (Liang et al., 2016).

The depth of insights from electrophysiological studies with these cells, however, is limited by their immature phenotype and cell-to-cell variability (Goversen et al., 2018b). Even genetically identical iPSC-CMs derived from the same donor display significant heterogeneity (Ma et al., 2011; Doss et al., 2012; Quach et al., 2018; Kernik et al., 2019; Akwaboah et al., 2021). Such shortcomings make it difficult to glean meaningful physiological information from patient-specific iPSC-CMs (Blinova et al., 2019), and have led to inconsistent results in multisite drug cardiotoxicity screening studies (Blinova et al., 2018). Developing an understanding of iPSC-CM heterogeneity is an essential step to improve the utility of these cells as a tool for use in precision medicine.

In recent years, extensive intercellular and intersubject variations in ion-channel expression patterns have been documented and motivated the development of cell-specific and heterogeneous populations of in silico models (Sarkar et al., 2012; Britton et al., 2013; Krogh-Madsen et al., 2016; Ni et al., 2018; Whittaker et al., 2020; Grandi et al., 2023; Lachaud et al., 2022; Kernik et al., 2019). Such modeling has identified electrophysiological features that increase the risk of proarrrhythmic events (Passini et al., 2017; Gong and Sobie, 2018; Ni et al., 2018).

While well-documented, here we argue that iPSC-CM electrophysiological heterogeneity is a pervasive and understudied issue. We use a combination of in vitro and in silico approaches and present a new method called rapid ionic current phenotyping (RICP) that makes it possible to correlate cell-specific ionic currents to AP morphology. The approach uses a recently published voltage clamp (VC) protocol from our lab (Clark et al., 2022) — the 10 s protocol provides insight into the presence and relative size of several key cardiac ionic currents. Using this data, we identify currents that influence AP morphology and explain both cell-to-cell variability and outliers in a population of iPSC-CMs.

We also use the short, 10 s, VC protocol data to draw the following conclusions about the cells used in this study:

- L-type calcium current (\(I_{\text{CaL}}\)) drives upstroke in iPSC-CMs with a depolarized AP morphology.
- Rapid delayed rectifier K\(^+\) current (\(I_{\text{Kr}}\)) plays a role in establishing the maximal diastolic potential.
- Seal-leak current contaminates VC recordings and contributes to a depolarized maximal diastolic potential.
- Large positive currents present at potentials \(>40\ \text{mV}\) correlate with a shortened AP duration.
- Several cells contain a strong outward current at 6 mV that is not present in computational AP models and correlates with AP duration.
2 Methods

2.1 iPSC-CM cell culture and electrophysiological setup

The in vitro data was previously published (Clark et al., 2022).

Frozen stocks of human iPSC-CMs were purchased from the Stanford Cardiovascular Institute Biobank. These cells were derived from an African-American female donor in a process approved by Stanford University Human Subjects Research Institutional Review Board. The use of cells from one donor allowed us to develop a method that aims to explain heterogeneity within a single individual, rather than considering effects at the population level.

Cells were prepared for electrophysiological experiments following the steps described in Clark et al. (2022). Briefly, cells were thawed and cultured as a monolayer in one well of a 6-well plate precoated with 1% Matrigel. Cells were cultured with RPMI media (Fisher/Corning 10-040-CM) containing 5% FBS and 2% B27 and kept in an incubator at 37°C, 5% CO₂, and 85% humidity. After 48 hours, cells were lifted with 1 mL Accutase, diluted to 100,000 cells/mL, and replated on 124 sterile 8 mm coverslips precoated with 1% Matrigel. Cells were cultured with RPMI media that was swapped every 48 hours. Cells were patched between days 5 and 15 after thaw.

Voltage clamp and current clamp recordings were acquired from 40 cells using the perforated patch technique (see Clark et al. (2022) for details). We excluded one cell from the analyses in this study because it had spontaneous alternans with inconsistent AP features. All cells had a pre-rupture seal of >300 MΩ.

2.2 Voltage clamp protocol

We have previously developed a voltage clamp protocol consisting of multiple short segments, each designed to isolate one key ionic current (Clark et al., 2022). The protocol was designed using optimization techniques and a mathematical model of iPSC-CMs (Kernik et al., 2019) to maximize, one at a time, the contribution to total current by each of seven key currents: I_Kr, I_CaL, sodium current (I_Na), transient outward K⁺ current (I_to), inward rectifier K⁺ current (I_K1), funny current (I_f), and slow delayed rectifier K⁺ current (I_Ks).

During our analysis, we found that the current measured 100 ms after a depolarizing step to 6 mV (I_{6mV}) was substantially different in many cells from that predicted by the mathematical model and we therefore included I_{6mV} as an 8th current measure.

For each current-isolating segment, we quantified the recorded total current I_{out} using either the minimum or the average over a 2 ms span centered at the following values (Figure 3C): I_{6mV} (600 ms, average), I_{Kr} (1262 ms, average), I_{CaL} (1986 ms, minimum), I_{Na} (2760 ms, minimum), I_{to} (3641 ms, average), I_{K1} (4300 ms, average), I_{f} (5840 ms, average), and I_{Ks} (9040 ms, average).
2.3 AP feature calculations

The transmembrane potential of each cell was recorded for 10 s. Of the 39 cells, 12 were not spontaneously beating. We computed a minimal potential (MP) for these non-spontaneous cells.

For cells that were spontaneously beating, we computed their MP (in this case, the minimum voltage during the AP), action potential duration at 90% repolarization (APD\(_{90}\)), cycle length (CL), and maximal upstroke velocity (dV/dt\(_{\text{max}}\)). The average of each feature was calculated for all cells that produced more than one AP during the 10 s recording.

2.4 iPSC-CM mathematical models

For comparison and to guide the analysis of our experimental data, we used two different mathematical models of iPSC-CM electrophysiology: the Paci et al. model (Paci et al., 2013) and the Kernik et al. model (Kernik et al., 2019). We set the cell capacitance (C\(_m\)) of these models to 45 pF, which is centrally located in the range (18-98 pF) of the capacitances for cells used in this study.

To avoid long transients and to better simulate our perforated patch experimental setup, we fixed intracellular sodium and potassium concentrations ([Na\(^+\)_i] and [K\(^+\)_i]) to their baseline steady state values (taken after 1000 s of spontaneous or paced current clamp simulation).

Because the leak through the imperfect pipette-membrane seal during single-cell patch-clamp experiments can substantially impact the electrophysiological recordings in these cells, we included a linear leak current (I\(_{\text{leak}}\)) in the mathematical models (Clark et al., 2023). We used a baseline value of 2 G\(\Omega\) for the seal resistance.

In addition to the seal-leak current, for voltage clamp simulations, we included explicit modeling of the following experimental artifacts: liquid junction potential offset (-2.8 mV), access resistance (20 M\(\Omega\)), and series resistance (R\(_s\)) compensation (70%), including supercharging (Lei et al., 2020; Lei, 2020).

2.5 Population of models and sensitivity analysis

A population of 500 individual models was generated from both the Paci and Kernik models with experimental artifact equations by randomly sampling conductances of I\(_{\text{Na}}\), I\(_{\text{Ca,L}}\), I\(_{\text{Kf}}\), I\(_{\text{Ko}}\), I\(_{\text{K1}}\), I\(_{\text{leak}}\), sodium calcium exchange current (I\(_{\text{NaCa}}\)), sodium potassium pump current (I\(_{\text{NaK}}\)), sodium background current (I\(_{\text{bNa}}\)), calcium background current (I\(_{\text{bCa}}\)), as well as C\(_m\) and R\(_s\) between 0.25 and 4x their baseline values.

We used a Spearman correlation to determine the sensitivity of the current-isolating time points to these parameters.

2.6 Linear regression

A linear least-squares regression was used to compare ionic currents and AP features for both the in vitro and the in silico data. A Spearman correlation coefficient and p-value were calculated for these data.
2.7 Software and simulations

Simulations were performed in Myokit v1.33.7 (Clerx et al., 2016). Additional analysis was done in Python using NumPy v1.21.6 and SciPy v1.7.3 (Virtanen et al., 2020).

All data, code and models can be accessed from GitHub (https://github.com/Christini-Lab/ap-vc-correlations.git).

3 Results

3.1 iPSC-CMs are heterogeneous

Of the 39 cells used in this study, 27 were spontaneously beating, and 12 were quiescent and depolarized (Figure 1). There is substantial cell-to-cell heterogeneity. Reporting the variation as the standard deviation (SD), we calculate a MP of $-52 \pm 10$ mV, action potential duration at 90% repolarization (APD$_{90}$) of $127 \pm 70$ ms, and maximum upstroke velocity (dV/dt$_{max}$) of $10.5 \pm 6.6$ V/s. Within the subset of spontaneously beating cells, there is additional heterogeneity in that some have a predictable and consistent CL, while the CL of others is highly variable (Figure S1).

![Figure 1: Current clamp recordings illustrate baseline heterogeneity.](https://example.com/figure1)

Figure 1: Current clamp recordings illustrate baseline heterogeneity. Spontaneously beating (n=27) and quiescent depolarized cells (n=12) from current clamp recordings of iPSC-CMs. If reported as mean $\pm$ SEM, the variation in MP ($-52 \pm 1.6$ mV), APD$_{90}$ (132 $\pm$ 14 ms), and dV/dt$_{max}$ (10.4 $\pm$ 1.2 V/s) appears quite small.

Large cell-to-cell variations in iPSC-CM AP data is common in the literature. Figure 2 shows the mean and SD...
error bars for MP, APD$_{90}$, and dV/dt$_{\text{max}}$ of 24 independent datasets taken from 14 studies. We selected these three AP features because they were the most consistently reported in manuscripts and have straightforward operational definitions. These data illustrate the large inter- and intralab heterogeneity present in iPSC-CM studies.

![Graph showing variations in AP features from 24 independent datasets.](image)

**Figure 2:** Variations in AP features from 24 independent datasets. The numbered studies on the right are independent datasets, named according to the first author (see references for full bibliographic information), and the numbers correspond to their position on the x-axis. Studies labeled with (N), (A), or (V) used AP features to sort cells into nodal, atrial, or ventricular groupings. The datasets from Lee et al. (2017) used differentiation protocols to induce either atrial-like (AI) or ventricular-like (VI) phenotypes. The datasets in this figure, including our own (position 5), are sorted in order from most to least depolarized MP.
3.2 Rapid ionic current phenotyping provides insight into AP outliers

As detailed in the Methods, we recently published a voltage clamp protocol (Clark et al., 2022) that was designed to isolate each of the following seven key ionic currents: $I_{Kr}$, $I_{CaL}$, $I_{Na}$, $I_{to}$, $I_{K1}$, $I_{f}$, and $I_{Ks}$. The protocol works by stepping to voltages designed to maximize the contribution of each current at different time points. Because of its short duration (10 s) and design to target multiple currents, we are calling this approach rapid ionic current phenotyping (RICP).

Figure 3A shows the voltage clamp protocol (top) and heterogeneity in ionic current responses (bottom) from the 39 cells included in this study. On the voltage clamp protocol, we have overlaid dashed lines to highlight current-isolating time points that we use to study ionic current dynamics. In addition to the seven designed current-isolating segments, we included another time point measure ($I_{6mV}$, the current recorded 100 ms after a depolarizing step to 6 mV), as we found that it provides insight into dynamics that are not present during other portions of the voltage clamp protocol.

Spontaneously beating cells are plotted in black and quiescent cells are in blue (Figure 3A-C). The pink spontaneous cell is an outlier, with an APD$_{90}$ value $>2x$ longer than the rest of the population.
Figure 3: VC variations and membrane potential in 39 cells. A, The optimized voltage clamp protocol (top), with lines overlaid that indicate time points designed to isolate each current. Responses to the voltage clamp protocol (bottom) for spontaneous (black), quiescent (blue), and the outlier (pink). B, APs from spontaneously beating cells (n=27). The pink AP is an outlier with an APD_{90} >2x longer than the rest of the population. C, Voltage recordings from quiescent and depolarized cells (n=12). The voltage recordings in B and C are the same as those shown in Figure 1 but are here separated for clarity.

Figure 4 shows the distribution of recorded total current values at each of the eight current-isolating time points. Similar to the AP data, we see a large variation in measurements during each of these current-isolating segments. Compared to the rest of the population, the outlier cell appears to have the smallest total current during the regions of the protocol designed to isolate I_{K1}, I_f, and I_{Ks}. All three of these currents conduct potassium ions that often contribute to repolarizing cardiomyocytes. This provides a possible explanation for how a cell with current measures at the tail ends of the distributions, can become an outlier in terms of an AP metric (e.g., APD), and shows how one may mechanistically explain outlier voltage dynamics in terms of the underlying ionic currents.
Figure 4: $I_{\text{out}}$ distribution at the eight current-isolating time points. Histograms display the distribution of recorded current values at each of the eight time points. The pink dashed line shows $I_{\text{out}}$ values for the outlier cell.
3.3 RICP identifies I\textsubscript{CaL} as driver of upstroke in depolarized cells

Figure 5A displays the relationship between the total current during each current-isolating segment and the dV/dt\textsubscript{max} for all cells. None of the eight current-isolating segments correlate with dV/dt\textsubscript{max}. Figure 4 shows the likely presence of I\textsubscript{Na} in many of these cells — we draw this conclusion because I\textsubscript{Na} is the only current expected to generate an I\textsubscript{out} of < -40 A/F within 2 ms after the I\textsubscript{Na} voltage step. However, the upstroke velocity is relatively small in most cells (Figure 2C) and does not correlate with the current measured I\textsubscript{Na}-isolating segment. The small upstroke velocities and the lack of correlation with I\textsubscript{Na} was an unexpected finding, given that I\textsubscript{Na} is present in many of these cells.

While there is no significant relationship with the VC segments, dV/dt\textsubscript{max} does correlate with MP (Figure 5B) — dV/dt\textsubscript{max} increases as MP becomes more hyperpolarized. The two cells with MP below -70 mV (denoted with red squares) stand out as having much larger upstroke velocities than the rest of the population. I\textsubscript{Na}, which is responsible for the upstroke in adult ventricular and atrial cardiomyocytes, recovers from inactivation at voltages below -65 mV. As such, one well-supported hypothesis for the larger dV/dt\textsubscript{max} in these two iPSC-CMs (Rook et al., 1999; Goversen et al., 2018b) is that they repolarize enough to make some sodium channels available for an I\textsubscript{Na}-driven upstroke. Other than having relatively hyperpolarized MP values, these two cells produce APs with few morphological similarities (Figure 5C).

Interestingly, when we exclude these cells from the regression analysis, and only consider iPSC-CMs with MP > -70 mV, a significant relationship emerges between the I\textsubscript{CaL}-isolating segment and dV/dt\textsubscript{max} (Figure 5D). This indicates that I\textsubscript{CaL} may be, at least partly, responsible for the upstroke in cells with MP > -70 mV.
Figure 5: \( I_{\text{CaL}} \) helps drive upstroke in depolarized iPSC-CMs. A, There is no correlation between \( I_{\text{out}} \) from the eight current-isolating segments and dV/dt\(_{\text{max}}\). B, dV/dt\(_{\text{max}}\) decreases as MP depolarizes. The red boxes denote the two cells with the most hyperpolarized MP and largest dV/dt\(_{\text{max}}\). C, The two highlighted cells from panel B share few AP commonalities with one another other than their relatively hyperpolarized MP and large dV/dt\(_{\text{max}}\). D, A trend emerges between \( I_{\text{out}} \) during the \( I_{\text{CaL}}\)-isolating segment and dV/dt\(_{\text{max}}\) when the two hyperpolarized cells are removed from the analysis.

3.4 RICP identifies \( I_{\text{Kr}}\)-isolating segment as predictor of MP

Four current-isolating segments (\( I_{\text{Kr}}, I_{\text{Na}}, I_{\text{K1}}, \) and \( I_{\text{f}} \)) of the VC protocol correlate with MP (Figure 6A), with the \( I_{\text{Kr}}\)-isolating segment being the strongest correlate (R=0.72). We selected cells at the two MP extremes to illustrate the relationship between the \( I_{\text{Kr}}\)-isolating segment and MP (Figure 6B): Cell 1 (blue) is the most hyperpolarized in the population (MP=-73 mV and APD\(_{90}=81\) ms) and Cell 2 is the most depolarized (quiescent with MP of -27 mV).

A plot of the \( I_{\text{Kr}}\)-isolating section of the VC protocol shows the voltage command (top) and current responses (bottom) for all cells in the population (Figure 6C). During this section of the protocol, cells were clamped to 6 mV for 750 ms, then stepped to -41 mV for 7 ms, and then back up to 9 mV. At the time point designed to isolate \( I_{\text{Kr}} \) (red dashed line), Cell 1 conducts a small positive total current (0.3 A/F) that increases in size, which
is consistent with $I_{Kr}$ recovering from rectification. In contrast, Cell 2 conducts a net negative current (-2.5 A/F) and does not show $I_{Kr}$ recovery characteristics, suggesting that this cell has much less $I_{Kr}$. These cells are examples of the population-level correlation seen between the $I_{Kr}$-isolating segment and MP. They indicate that $I_{Kr}$, and other currents that may be present during the step to -40 mV, likely play a critical role in establishing MP in these cells.

**Figure 6:** Correlations between ionic current segments and MP. A, $I_{out}$ from the $I_{Kr}$, $I_{to}$, $I_{K1}$, $I_{r}$, and $I_{Ks}$ segments correlate significantly ($p<.05$) with MP. B, Current clamp recordings from all cells. The iPSC-CMs with the most hyperpolarized (blue, Cell 1) and depolarized (green, Cell 2) MP values are compared. C, Traces from the $I_{Kr}$-isolating segment of the VC protocol for all cells. The VC protocol (top) includes a 750 ms prestep at 6 mV, followed by a 7 ms step at -41 mV, and then a step to 9 mV. The VC protocol was designed to isolate $I_{Kr}$ at 1262 ms (red dashed line). Cell 1 (blue) and Cell 2 (green) are highlighted.
We used an iPSC-CM mathematical model with a linear seal-leak current (see Methods) to further study the potential role of $I_{Kr}$ in establishing a MP. Figure 7 shows the effect of scaling $I_{Kr}$ conductance ($g_{Kr}$) on the MP for a model with a 2 GΩ seal resistance. While holding all other parameters constant, reducing $g_{Kr}$ by >70% of baseline results in substantial depolarization. The 10% to 90% range of our experimental data corresponds to a roughly 70% to 85% reduction in $g_{Kr}$ (Figure 7B). These model findings are consistent with the correlations seen between the experimental $I_{Kr}$-isolating $I_{out}$ measurements and MP.

Figure 7: AP simulations of a Kernik-Clancy model with a 2 GΩ seal resistance and varying levels of $g_{Kr}$. A, APs generated by running the model with varying levels of $g_{Kr}$ from 0.1 to 1.6 times the baseline Kernik-Clancy value (0.218025 nS/pF). The arrow indicates that depolarized cells have less $I_{Kr}$ (i.e., smaller $g_{Kr}$). B, Relationship between $g_{Kr}$ and MP for cells plotted in A. The yellow highlighted region represents the 10% to 90% range of MP values from cells in this study.

3.5 Seal-leak current likely contributes to the depolarized MP

In addition to the $I_{Kr}$-isolating segment, the $I_{to}$, $I_{K1}$, $I_{f}$, and $I_{Ks}$-isolating segments also weakly correlate with MP (Figure 6A). Some of these correlations are unexpected and counter-intuitive as, e.g., $I_{to}$ is typically inactivated during phases 3 and 4 of the AP. We hypothesized that these correlations may be influenced by a leak artifact current caused by an imperfect seal between the pipette tip and cell membrane — we recently demonstrated that this leak current, present at voltages far from zero, can substantially depolarize the MP of iPSC-CMs (Clark et al., 2023).

In an attempt to investigate the role of $I_{leak}$ on these segments, we developed a population of Paci models that include patch-clamp experimental artifact equations (Figure 8). In this in silico population, the $I_{Kr}$ segment is also the main correlate with MP, with $I_{to}$, $I_{Ks}$, and $I_{f}$, being more weakly correlated with MP (figure S3).

A sensitivity analysis of the population of models shows that the segments weakly correlating with MP in the experiments (i.e., the $I_{to}$, $I_{K1}$, $I_{f}$, and $I_{Ks}$ segments) are all sensitive to the conductance of $I_{leak}$ (i.e., $g_{leak}$, Figure
9). $I_{\text{leak}}$ is modeled as a linear current with a reversal potential of zero, and so it will increase when cells are clamped to voltages that are far from 0 mV. Seeing as $I_{K_s}$ and $I_{to}$ are elicited by stepping to large positive voltages and $I_{K1}$ and $I_f$ to large negative voltages, this is consistent with these segments being contaminated by $I_{\text{leak}}$. Increased $g_{\text{leak}}$ increases the total (net outward) currents computed at positive voltages (e.g., $I_{K_s}$ and $I_{to}$ segments) and depolarizes the MP, consistent with the positive correlation seen between MP and the $I_{K_s}$ and $I_{to}$ segments in our experiments. Similarly, the negative correlations between MP and $I_{K1}$ and $I_f$ in our cells is consistent with the in silico results where increased $g_{\text{leak}}$ during these hyperpolarized segments in the models contribute to the large (net inward) current and depolarized MP values.

Figure 8: **A population of Paci models compared to experimental data.** 500 Paci individuals were generated by varying ionic current conductances and experimental artifact parameters (see Methods). The dashed orange line shows the average $I_{\text{out}}$ for all models, and the shaded region is the range of values for all models. The experimental data are displayed in gray.
Correlations calculated between model parameter values (primarily conductances; e.g., $g_{Kr}$) and $I_{out}$ during each of these four segments. Each current is sensitive to its own conductance, with outward currents ($I_{to}$ and $I_{Ks}$) having a positive correlation and inward currents ($I_{K1}$ and $I_{f}$) showing as a negative correlation to their own conductance. Each current is also sensitive to $g_{leak}$. Both $I_{to}$ and $I_{Ks}$ have a surprisingly strong sensitivity to $g_{NaCa}$.

Figure 9: $I_{to}$, $I_{K1}$, $I_{f}$, and $I_{Ks}$-isolating segments are sensitive to seal-leak current. Correlations calculated between model parameter values (primarily conductances; e.g., $g_{Kr}$) and $I_{out}$ during each of these four segments. Each current is sensitive to its own conductance, with outward currents ($I_{to}$ and $I_{Ks}$) having a positive correlation and inward currents ($I_{K1}$ and $I_{f}$) showing as a negative correlation to their own conductance. Each current is also sensitive to $g_{leak}$. Both $I_{to}$ and $I_{Ks}$ have a surprisingly strong sensitivity to $g_{NaCa}$.

3.6 RICP identifies strong outward currents as drivers of APD$_{90}$

Four segments ($I_{6mV}$, $I_{K1}$, $I_{f}$, and $I_{Ks}$) of the VC protocol correlate with APD$_{90}$ (Figure 10A). Three of the segments ($I_{K1}$, $I_{f}$, $I_{Ks}$) were designed to isolate potassium-conducting currents, each of which are clamped to voltages far from 0 mV. The $I_{6mV}$ segment was added to quantify observed current dynamics different from those produced by the mathematical models and which we hypothesized could be explanatory of AP morphological differences.

To illustrate the relationship between currents and APD$_{90}$, we investigated the current responses from two different cells (Figure 10B): one with the shortest APD$_{90}$ (Cell 3) and one with the second longest APD$_{90}$ (Cell 4).

The strong correlation between the $I_{Ks}$-isolating segment and APD$_{90}$ indicates that the net outward current at large positive voltages affects APD$_{90}$. Cell 3 has a slightly larger outward current during this $I_{Ks}$-isolating segment when compared to Cell 4 (Figure 10D).

$I_{f}$ and $I_{K1}$-isolating segments correlate less strongly with APD$_{90}$ and in the opposite direction than what would be expected. While both of these currents are expected to have an effect on AP duration, their current-isolating segments are likely contaminated with $I_{leak}$ (Figure 9) that is contributing to the opposite direction of this (Clark et al., 2023).
The outward current present during the $I_{6mV}$ segment is much larger in Cell 1 than any other in the population, and is very likely driving the rapid repolarization shown in this cell. In contrast, Cell 2 has a nearly balanced net current during the $I_{6mV}$ segment. In an attempt to understand the dynamics at play, we used our population of models to study the ionic currents that may be contributing to total current during this segment.

Figure 11 shows experimental current responses (gray) and the range of traces generated from the population of models during the $I_{6mV}$ step (600 ms). The experimental responses for many of the cells (11/39) during this $I_{6mV}$ segment are more positive than any individual in the model population. This data indicates that several of these cells have a strong repolarizing ionic current that is not represented in the iPSC-CM electrophysiological...
model.

Figure 11: Comparison of Paci and experimental $I_{6mV}$. Experimental (gray) and range of Paci model traces (shaded orange) during $I_{6mV}$ voltage step.

4 Discussion

iPSC-CM heterogeneity is an understudied issue that confounds experimental results. Here, we propose RICP as a novel approach to study the ionic current underpinnings of AP heterogeneity. By analyzing data from a brief, optimized 10 s VC protocol, we generate the following insights about the iPSC-CMs used in this study:

- $I_{CaL}$ is partly responsible for driving upstroke when MP is $>-70$ mV
- $I_{Kr}$ contributes to establishing the MP
- $I_{leak}$ contaminates VC responses at very positive and negative voltages and contributes to MP
- Strong outward current elicited in the range of $I_{Ks}$ activation correlates with AP duration
- These cells have an unidentified outward current present at 6 mV that is not recapitulated by iPSC-CM mathematical models

Overall, this study demonstrates both the large cell-to-cell heterogeneity in iPSC-CMs and provides a tractable method to study the ionic current determinants of such variability during patch clamp experiments.
4.1 iPSC-CM heterogeneity confounds experimental results and limits reproducibility

The utility of iPSC-CMs in arrhythmia research has long been limited by their heterogeneous and unpredictable phenotype. The inherent electrophysiological variation of these cells makes it difficult to measure consistent population-level signals. The results can be confounding. For example, Blinova et al. (2019) show how iPSC-CMs purchased from the same vendor can produce opposite results to the same drug treatment protocol applied in different labs. The same group also conducted a comparative study of iPSC-CMs derived from multiple healthy individuals and showed no obvious patient-specific characteristics, likely due to the inherent heterogeneity of the cells (Blinova et al., 2019). Unfortunately, little is known about the sources or ionic current underpinnings that lead to such inconsistent results.

Shortly after the development of iPSC-CMs (Zhang et al., 2009), investigators began to sort them into nodal, atrial, and ventricular groups based on their AP features (Ma et al., 2011; Moretti et al., 2010; Itzhaki et al., 2011). The resultant sub-groups have less variance in AP morphology than the entire population of cells; i.e., sub-grouping reduces the apparent population heterogeneity. It has since been shown that, when groupings are ignored, iPSC-CM AP features are normally distributed (Du et al., 2015), leading some to argue that iPSC-CM chamber-specific grouping is a fallacy (Kane et al., 2016). While there was some initial push-back (Giles and Noble, 2016), evidence has since accumulated that indicates AP-based chamber specifications can be reductive and misleading. For example, individual iPSC-CMs do not have canonical gene expression profiles and are sensitive to noncanonical modulators of cell fate, pointing towards a physiology that is not seen in primary cardiomyocytes (Biendarra-Tiegs et al., 2019). Similarly, gene expression and phenotype data acquired from iPSC-CMs has shown a combination of attributes in a mixed population that is not consistent with any one chamber (Schmid et al., 2021).

iPSC-CM heterogeneity has long been viewed as a problem, which has led to much research into optimizing differentiation protocols to produce consistent, largely homogeneous cellular populations for in vitro cardiotoxicity studies (Blinova et al., 2018). We believe these characteristics of iPSC-CMs should be reframed, not as a problem that needs to be solved, but as biological reality that can serve as a laboratory model of cardiomyocyte heterogeneity.

It is well known that the AP morphology of primary cardiomyocytes isolated from the same regions of the of the heart can vary widely (Lachaud et al., 2022; Zaniboni et al., 2000). Lachaud et al. (2022) isolated 150 cells from a single rabbit left ventricle and, using optical recordings, showed that the APD$_{90}$ ranged from 100 to >300 ms. While these primary cells specifically did not possess the multi-chamber molecular expression heterogeneity (Moretti et al., 2010) seen in iPSC-CMs, such results still indicate that substantial cell-to-cell variation in AP morphology is ubiquitous. iPSC-CMs have the potential to serve as a valuable tool to study and develop methods to help us understand electrophysiological heterogeneity with the hope of surfacing physiologically relevant patterns. We believe the work presented here serves as a step in this direction.
4.2 RICP is a tool to understand ionic current mechanisms of iPSC-CM heterogeneity

We used our recently published VC protocol to capture a rich snapshot of current dynamics that is explanatory of iPSC-CM AP morphology. This 10 s protocol provided information about several currents (e.g., $I_{CaL}$, $I_{Kr}$) and their effects on AP morphology ($dV/dt_{max}$, MP). Other studies have collected VC data for one or two currents and shown correlations with certain AP morphological features from the same cells (Feyen et al., 2020; Schmid et al., 2020). However, we believe this is the first study that attempts to collect information about several currents with a <10 s protocol and use this data to explain AP features.

The VC protocol was designed to identify the presence and relative size of seven individual currents, but each of the segments is contaminated, at least to some extent, by off-target currents. For example, $I_{leak}$ contaminates every segment of the protocol, as it contributes current at all voltages, with increased $I_{leak}$ at voltages far from 0 mV. The sensitivity analysis shows how the $I_{K1}$, $I_{to}$, $I_{f}$, and $I_{Ks}$ segments, all of which are clamped far from 0 mV, are sensitive to changes in the seal-leak resistance (Figure 9). Some currents (e.g., $I_{Na}$) have distinct dynamics that provide for relatively easy isolation, but others (e.g., $I_{Kr}$) always open when several currents conduct ions.

The isolation of currents could potentially be improved through the development of new VC protocols with different optimization methods or by using new computational models that better capture the dynamics of iPSC-CMs used in a specific experimental context. However, we believe new protocols will only result in marginal improvements. Instead, we think there is far more to gain by developing methods to tease apart contributions from the different currents. Depending on the objective of the experiments, one could sequentially dissect current contributions (Banyasz et al., 2011) or fit computational models to individual traces (Groenendaal et al., 2015; Lei et al., 2017) — either of which could provide estimates of cell-specific ionic current densities.

4.3 $I_{CaL}$ likely drives upstroke in many iPSC-CM studies

Using RICP we identified $I_{CaL}$ as a contributor to upstroke velocity in the depolarized iPSC-CMs used in this study. Despite the presence of $I_{Na}$ in most of our cells, $I_{CaL}$ appears to be the predominant driver of upstroke. This is likely because, at depolarized MP values (e.g., $> -70 \text{ mV}$), $I_{Na}$ is inactive and cannot generate an inward current to drive upstroke.

This implies a mechanism similar to AP generation in SA nodal cells — despite the presence of $I_{Na}$ (Verkerk et al., 2009), the upstroke is primarily driven by calcium currents (Vinogradova et al., 2000). This finding also hints that $I_{CaL}$ as the likely driver of upstroke in a few of the studies in Figure 2 where $dV/dt_{max}$ is $< 20 \text{ V/s}$ — e.g., the “atrial” cells from Es-Salah-Lamoureux et al. (2016). As we, and others have shown, it is possible to recover a polarized MP by dynamically clamping a synthetic $I_{K1}$ (Goversen et al., 2018a; Becker et al., 2020; Clark et al., 2022). Such an approach makes it possible for $I_{Na}$, among other currents, to recover from inactivation and results in APs with a faster upstroke velocity and more mature appearance.
4.4 $I_{Kr}$ is likely an important current in establishing MP in depolarized iPSC-CMs

$I_{K1}$ conductance is often thought to be reduced in iPSC-CMs relative to adult cardiomyocytes, resulting in the depolarized MP. However, iPSC-CMs can have large amounts of $I_{K1}$, and yet, still be depolarized compared to adult cells (Horváth et al., 2018). One hypothesis for this discrepancy in MP between adult and iPSC cardiomyocytes is the increased role of $I_{leak}$ in iPSC-CMs (Horváth et al., 2018; Clark et al., 2023) caused by their smaller size relative to adult cardiomyocytes.

In this study, we identify $I_{Kr}$ as likely playing a role in establishing the MP of iPSC-CMs. The correlation between $I_{Kr}$ and MP is unlikely to be substantially influenced by leak as the VC step to isolate $I_{Kr}$ is close to the expected seal leak reversal potential. In both our experimental data and in populations of models based on either the Paci or Kernik models, the $I_{Kr}$-isolating segment has the strongest correlation to MP (-0.66, -0.54, and -0.58 for experimental data, Paci, and Kernik, respectively, Figures 6, S3 and S4).

This finding is in agreement with previous work showing that iPSC-CM MP values are sensitive to E-4031 (Doss et al., 2012). Our modeling work indicates that with a small $I_{leak}$ (2 GΩ seal) and substantial reduction in baseline $I_{Kr}$, the model will depolarize to MP values similar to those shown in our study.

On a related note, $I_{Kr}$ appears to be absent or significantly reduced when iPSC-CMs are prepared for experimentation in automated patch-clamp systems (Ismaili et al., 2023) — we hypothesize that this lack of repolarizing current may be directly responsible for the depolarized MP and lack of spontaneity in iPSC-CMs used with automated patch-clamp systems cells (Li et al., 2019).

4.5 The optimized VC protocol elicits a large unidentified outward current at 6 mV in some cells

One of the core features of RICP is that we use a noncanonical VC protocol to quickly probe a wide range of system dynamics. Most electrophysiological experiments, on the other hand, are designed with well-established VC protocols to study one current at a time. Such studies are effective at characterizing the magnitude and kinetics of target ionic currents. These protocols are, by design, reductive in nature. They attempt to extract information about a component part (the ion channel) from the broader dynamic system. As a result, these protocols are far less likely to identify unexpected system behaviors arising outside of that target current.

We identified a region of the protocol (stepping from -80 mV to 6 mV) that contributed information not captured in other current-isolating segments. While we are unable to identify the current present in a subset of cells during the $I_{6mV}$ time point (Figure 10C), it appears to correlate with a shortened APD$_{90}$. The computational models are unable to capture these dynamics, and so the mechanism remains unclear.

This could be a non-cardiac current — Horváth et al. (2020) previously identified the presence of the non-cardiac big conductance $Ca^{2+}$-activated $K^+$ current ($I_{BK,Ca}$) in their cells. They suggest that this could have been introduced by genetic alterations (Kilpinen et al., 2017) that can occur during cell differentiation and culture. While we think this is an unlikely explanation for the current we see in our cells, it is a plausible hypothesis and...
provides a potential mechanism that may play a role in iPSC-CM heterogeneity that can lead to reproducibility issues.

4.6 Beat-to-beat variability and ionic determinants of cycle length

We did not see a significant correlation between any current-isolating element and CL (Figure S2). There was a tendency towards more current in the \( I_f \)-isolating segment and shorter CL, as seen in the Paci-based population of models (Figure S3), but this did not meet the threshold for statistical significance \((p<.05)\).

Investigation of correlations between CL and underlying ionic currents is made difficult by the substantial beat-to-beat variability of CL in some of our cells (Figure S1). The average coefficient of variation for cycle length is 13.8% while for APD\(_{90}\) it is only 3.3% and 7.1% for \( dV/dt_{\text{max}} \). For comparison, (rabbit) sinoatrial nodal cells beat much more regularly (with a coefficient of variation of \( 2.0\% \) CL (Wilders and Jongsma, 1993)), as do embryonic chick ventricular cells (3.9% (Krogh-Madsen et al., 2017)). The mechanism of why some iPSC-CMs have such irregular cycle lengths with a slow and erratic diastolic phase, rather than a steady depolarization to a threshold for AP take-off, is not is not clear.

4.7 Limitations and future directions

The RICP method shows how a brief VC protocol can be used to provide mechanistic insights into AP morphology and heterogeneity. This protocol, however, does not perfectly isolate each of the seven currents, and so the causal relationships of currents with AP morphological features is likely weaker than if we conducted traditional drug block experiments. In the future, we believe methods that tease apart the current contributions at each time point has the potential to improve insights drawn from this VC protocol, such as ion channel co-expression patterns.

In this study, we focus on a set of cells from a single differentiation batch. While this highlighted the extent of heterogeneity within cells that should be very similar, in the future it would be interesting to conduct this RICP approach on cells from multiple donors, across multiple differentiation batches, and in multiple laboratory settings.

4.8 Conclusion

We believe that RICP has the potential to improve our understanding of iPSC-CM heterogeneity. The brief duration and targeting of most key cardiac ionic currents make this protocol a valuable tool in the study of iPSC-CM AP heterogeneity and drug mechanisms. If all single-cell patch-clamp iPSC-CM experiments collected this type of data, we believe the field would have a better grasp on the ionic current underpinnings of inter- and intralab heterogeneity.
5 Disclosures

The authors declare that they have no competing interests.

6 Author Contributions

A.P.C. designed research, analyzed data, prepared figures, and drafted manuscript. A.P.C. and S.W. performed experiments. A.P.C. and K.E.F. completed the sensitivity analysis. A.P.C., K.E.F., T.K.M., and D.J.C. interpreted results. T.K.M. and D.J.C. edited and revised manuscript. All authors approved final version of manuscript.

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